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ANTÓNIO EDUARDO BRUNO LOPES JOÃO

Orientadores: Professora Doutora Maria de São José Garcia Alexandre

Professor Doutor Carlos Penha Gonçalves

Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na especialidade de Segurança Alimentar

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This work is dedicated to my parents.

Acknowledgments

I want to acknowledge all the people that directly or indirectly have contributed to the accomplishment of this work. I feel deeply honored and grateful to all those that have helped me in this long and hard journey. It is a unique moment in my professional life and it was made possible by all persons with whom I have worked and it is with a feeling of great respect and gratitude that I want to address them here.

First of all, I would like to thank Professor Maria de São José, for all the support, the patience and the inspiration in the development of this thesis. It was following a lecture by Professor Maria de São José that the idea came out. The passionate way the lecture was given was really motivational and inspiring. Professor Maria de São José was paramount for all the work that followed, with a special note for the publication of the papers. I wish to thank deeply to Colonel Veterinary Penha Gonçalves, my former chief, for all the stimulus for pursuing this academic study and for the example of well doing, knowledge, intelligence and wisdom. It was for me, really a privilege to take part on the working team of Colonel Veterinary Penha Gonçalves. My truly thanks for Professor João Mesquita who was tireless in the processes of writing and submission of the papers, always with a gentle and good mood and for Professor Ana Duarte for the kindness and motivating words that always gave me.

I feel a special sense of gratitude to Doctor Mónica Oleastro for the work of genotyping of the viral strains, for the wise advising, the intelligent remarks, the good conversations and the prompt and friendly response to my requests. I also want to thank in a particular way Doctor Rita Sousa for the work of genotyping, the kindness and the noroviruses genetic diversity book who was a source of inspiration in the writing process and to MSc Inês Costa for the contribution in the writing of the papers.

For me it has been a special honor and privilege to work during all these years with the team of persons of the Portuguese Army, my Comrades of Arms, that were very important in this process, so to Major Med Vet Júlio Carvalho, Major Med Vet José Freitas, Major Med Vet Pedro Silva, Major Med Vet Wilson Antunes, Major Med Vet Carlos Alonso and Captain Med Vet Inês Gomes I thank you all very much. My true gratitude to First Lieutenant Susana Rodrigues, Second Lieutenant Joana Fernandes, Second Lieutenant Octávio Barbosa, Second Lieutenant Nuno Bem, Sargent Vanda

Queimada, Sargent Carina Gomes, Sargent Ricardo Vieira, Soldier Tiago Pereira and MSc Verónica Ribeiro.

I thank all the other personnel of the Laboratorial Military Unit, namely Lieutenant Colonel Farm Vânia Tira-Picos, Major Farm Miguel Cabrita, Captain Farm Tiago Gonçalves, Captain Farm Pedro Neto, Captain Farm Paula Lopes, Second Lieutenant Luís Carvalho, Sargent Licínia Gomes, Soldier João Castela and MSc Joana Saramago.

To all the people who directly or indirectly helped me in the work of collecting epidemiologic data I am really grateful: Lieutenant Colonel Med Vet Isabel Gabriel, Lieutenant Colonel Med Vet Daniel Simões, Lieutenant Colonel Med Vet Ana Silva, Major Med Vet António Coimbra, Major Med Vet Samuel Miguéis, Captain Med Vet João Machado, Second Lieutenant Med Vet João Cabrita, Sargent-Chief José Simões, MSc Cristina Orrico and Soldier André Assis.

With the risk of forgetting the names of many persons in the Army that contributed to this study to the doctors, nurses, pharmacists and health technicians in the health centers and in the hospitals, I thank you all very much.

A special thanks to Doctor Ana Rita Henriques, my doctorate colleague and close friend for the support and the stimuli to write this thesis.

To my close friends for all the patience and unconditional support. It is my privilege to have you nearby.

Finally, to the best Family a person can have my infinite gratitude.

Financial Support Acknowledgements

The Present work was partially supported by the Portuguese Army.

The author wants to acknowledge the Portuguese Army for the conditions provided for the experimental work, the development of the thesis and the financial support.

Resumo

Surtos de gastrenterite por norovírus no Exército Português

As infeções gastrointestinais encontram-se entre as doenças mais frequentemente reportadas como causa de diminuição da capacidade operacional e da prontidão de uma força militar. Os norovírus são os agentes mais vezes implicados na doença aguda gastrointestinal em teatros de operações, marítimos e terrestres, e em forças estacionadas ou centros de treino militares das Forças Armadas de Países Ocidentais.

Esta tese teve como principal objetivo avaliar a relevância da gastrenterite aguda causada por norovírus no Exército Português. Foi estabelecido um sistema de vigilância no Exército, de modo que todos os surtos de gastroenterite são reportados ao Laboratório de Bromatologia e Defesa Biológica. Assim, verificou-se que entre 2013 e 2017 foram registados 14 episódios, que afetaram cerca de 410 militares, tendo os norovírus sido responsáveis pela maioria dos surtos (8/14) e dos casos (210/410). Os surtos ocorreram em exercícios militares e em bases do Exército com impacto na capacidade operacional e funcionamento regular das Unidades. Embora a origem destes tenha sido difícil de confirmar, acredita-se que 5 (63%) tiveram origem alimentar (incluindo os manipuladores de alimentos e a água) e que em 3 (37%) predominou a transmissão pessoa a pessoa. Os surtos foram causados por norovírus dos genogrupos (G) I e II, contudo o G II foi responsável pelo maior número de surtos (6/8) e casos (145/210). O genogrupo I esteve associado à água e o genogrupo II à transmissão pessoa a pessoa ou aos alimentos. Cada surto foi causado por um genótipo distinto, refletindo a grande diversidade genética dos norovírus que se encontram em circulação. Três dos genótipos identificados (GI.9, GII.17 e GII.16-GII.2) foram reportados pela primeira vez em Portugal.

Ao identificar o norovírus como o mais importante agente etiológico de surtos de gastroenterite aguda e de doença com origem nos alimentos e na água no Exército Português, o presente trabalho contribuiu para a alteração de medidas preventivas de doenças de origem alimentar e para o reforço de ações de controlo dos surtos causados por este agente.

Palavras-chave: Norovírus, Exército Português, surtos de gastroenterite, sistema de vigilância e doença de origem alimentar

Abstract

Norovirus outbreaks in the Portuguese Army

Gastrointestinal infections have consistently been among the most frequent diseases and non-battle injuries, degrading operational effectiveness and force readiness in the military. Noroviruses appear as the most frequent agents causing acute gastrointestinal illness both in maritime and land theatres of operations, as well as in stationed troops or military training centers in many western countries.

Nevertheless, little is known on the burden of norovirus gastroenteritis in the Portuguese military. In this work we set out to establish a gastroenteritis outbreak surveillance system (GOSS) to report disease cases to the Bromatology and Biologic Defense Laboratory during the five-year period, 2013-2017. During this period 14 gastroenteritis outbreaks were registered that affected a total of 410 military. Noroviruses were responsible for the majority of the outbreaks (8/14) and disease cases (210/410). Norovirus outbreaks occurred either in military exercises or in military bases and showed to have an impact on force readiness and operational effectiveness. In most cases the origin of the outbreaks was difficult to confirm but five (63%) were likely foodborne (including food handlers) or waterborne and three (37%) had predominant person-to-person transmission. Outbreaks were caused by both genogroup (G) I and II, but G II clearly outnumber those caused by G I (6/8) and case numbers (145/210). Norovirus GI was associated with waterborne outbreaks while GII was associated to foodborne and person-to-person transmission. Each outbreak was caused by a different genotype highlighting the high genetic diversity of the circulating noroviruses. Three of the identified genotypes (GI.9, GII.17 and GII.16-GII.2) were reported for the first time in Portugal.

In conclusion, the present thesis identified norovirus as the most important etiologic agent of acute gastroenteritis outbreaks and the most frequent cause of food- and waterborne illness in the Portuguese Army. This work had contributed to change preventive measures and allowed the reinforcement of control actions that minimized the impact of norovirus outbreaks.

keywords:

Norovirus, Portuguese Army, gastroenteritis outbreak, surveillance system, foodborne disease

The results presented in this thesis have been published:

Papers in International peer-reviewed journals

Lopes-João A, Mesquita JR, de Sousa R, Oleastro M, Penha-Gonçalves C, Nascimento MS. (2019). A simultaneous norovirus outbreak in three Portuguese Army bases in the Lisbon region, December 2017. J R Army Med Corps. **0**:1-4.

Lopes-João A, Mesquita JR, de Sousa R, Oleastro M, Penha-Gonçalves C, Nascimento MS. (2018) Country-wide surveillance of norovirus outbreaks in the Portuguese Army, 2015-2017. J R Army Med Corps. **164** (6):419-422.

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Lopes-João A. (2013) Surtos de Toxinfecção Alimentar no Exército Português 2006-2011. Revista Portuguesa de Ciências Veterinárias. **108** (587-588) 154-160.

Communications in congress and scientific meetings

Oral communications

Lopes-João A, Mesquita JR, de Sousa R, Oleastro M, Penha-Gonçalves C, Nascimento MS. A countrywide surveillance of norovirus outbreaks in the Portuguese Army (2018) in "Food Defense International Symposium", 11 September, Academia Militar, Amadora, Portugal.

Lopes-João A, Mesquita JR, de Sousa R, Oleastro M, Penha-Gonçalves C, Nascimento MS. Toxinfeções alimentares no Exército, a experiência do Laboratório de Bromatologia e de Defesa Biológica (2018) in "Doenças de transmissão alimentar e saúde dos manipuladores", 22 February, Centro de Saúde Militar de Coimbra, Portugal.

Lopes-João A, Mesquita JR, de Sousa R, Oleastro M, Penha-Gonçalves C, Nascimento MS. Toxinfecçoes alimentares no Exército Português (2015) in VI Seminário de Alimentação das Forças Armadas, Defesa e Cultura Alimentar, 28 October, Rio de Janeiro, Brazil.

Lopes João A, Mesquita JR, Costa I, Oleastro M, Nascimento MS. Surto de gastroenterite por norovirus num Exercício Militar do Exército Português (2014) in VI Congresso da Sociedade Portuguesa de Ciências veterinárias, about veterinary sciences: praxis and future, 4 April, Instituto Nacional de Investigação Agrária e Veterinária, Oeiras, Portugal.

Lopes João A, Mesquita JR, Costa I, Oleastro M, Nascimento MS. Norovirus, um agente emergente (2013) in VIII Congresso Científico AEFFUP, about infectious emergent disease, 27 November, Faculdade de Farmácia da Universidade do Porto, Porto, Portugal.

Posters

Lopes João A, Mesquita JR, Costa I; Oleastro M, Nascimento MS (2014) Toxinfeções alimentares no Exército Português 2006-2013. VI Congresso da Sociedade Portuguesa de Ciências veterinárias about veterinary sciences: praxis and future", Instituto Nacional de Investigação Agrária e Veterinária, Oeiras, Portugal 3-5 April.

Lopes João A, Mesquita JR, Costa I; Oleastro M, Nascimento MS (2013) Norovirus gastroenteritis in a nurse attending an acute gastroenteritis outbreak in a care facility of the Portuguese Armed Forces. IV Congresso Nacional de Virologia e VIII Encontro Nacional da Sociedade Portuguesa de Virologia, Lisboa, Portugal 25-26 November.

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ABHR	Alcohol-based hand rub
AHC	Army Health Center
AHD	Army Health Direction
BBDL	Bromatology and Biologic Defense Laboratory
BCDLU	Biologic and Chemical Defense Laboratory Unit
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CDC	Center for Disease Control and prevention
CEN	European Committee for Standardization
cfu	Colony Forming Unit
E. coli	*Escherichia coli*
ECDC	European Center for Disease Control and Prevention
EFSA	European Food Safety Authority
EIA	Enzyme immunoassay
EST	Epidemiologic Surveillance Team
FTA	Flinders Technology Associates
FUT	Fucosiltransferase
GEC	Genomic Equivalent Copies
GOSS	Gastroenteritis Outbreak Surveillance System
GI	Genogroup I
GII	Genogroup II
HACCP	Hazzard Analysis and Critical control Points
HBGA	Histoblood group antigens
HNORS	Hospital norovirus outbreak reporting scheme
IFN	Interferon
IL	Interleukin
ISO	International Organization for Standardization
MVU	Military Veterinary Unit
NGS	Next generation sequencing
Norostat	Norovirus Sentinel Testing and Tracking
NORS	National Outbreak Report System
NoV	Norovirus
nm	nanometer
ORF	Open reading frame

P22	Protein 22
P48	Protein 48
P-domain	Protruding domain
PCR	Polymerase chain reaction
POL	Polymerase
ppm	Parts per million
PRO	Protease
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
RT-qPCR	Reverse transcription quantitative polymerase chain reaction
S-domain	Shell-domain
SRSV	Small round structured virus
spp.	species
STEC	Shigatoxinogenic *Escherichia coli*
TNF-α	Tumor necrosis factor alfa
UK	United Kingdom
USA	United States of America
VLP	Virus-like particle
VPg	Viral protein genome-linked
VP1	Major capsid protein
VP2	Minor capsid protein
WHO	World Health Organization

I. INTRODUCTION

1. Norovirus

Human noroviruses are the leading cause of epidemic and sporadic gastroenteritis across all age groups (Glass et al. 2009; Robilotti et al. 2015). Noroviruses are ubiquitous, associated with 18% of acute gastroenteritis cases worldwide, with similar proportions of disease in high-, middle-, and low- income settings (Ahmed et al. 2014). Noroviruses are estimated to cause approximately 200.000 deaths annually worldwide, with about 70,000–100,000 fatal cases among children in developing countries (Ahmed et al. 2014; Lopman et al. 2016). Noroviruses are highly contagious and humans are the only known reservoir for human noroviruses (Green 2013). Transmission is fecal-oral and occurs via direct person-to-person, foodborne, waterborne or through fecal contamination or environmental fomites (Green 2013).

1.1. The discovery of norovirus

In 1929, Zahorsky was the first that described, what is thought to be norovirus gastroenteritis, “hyperemesis hemis” or “winter vomiting disease”, an illness characterized by the sudden onset of self-limited vomiting and diarrhea that typically peaked during the colder months (Patel et al. 2009).

During the 1940-1950s, several studies demonstrated the transmission of this “gastroenteric disease” to healthy volunteers by oral administration of a bacteria-free stool filtrate (Kapikian 2000). Because the infectious agent was transmissible after filtration, a viral etiology was at that time suspected (Kapikian 2000). In 1972, Kapikian identified a 27-nm virus-like particle, by immuno-electron microscopy, in an infectious stool filtrate from an outbreak of gastroenteritis that occurred in 1968 in an elementary school in Norwalk, Ohio (Kapikian et al. 1972). This allowed the recognition and identification of a virus-like particle that did not have a distinctive morphology and was among the smallest viruses known as the cause of the Norwalk outbreak (Kapikian 2000).

Over the two decades following the discovery of Norwalk virus, viruses similar to Norwalk virus in morphology and associated with similar clinical manifestations, but antigenically distinct, were discovered and provisionally classified as “small round structured viruses” (SRSV) (Dolin et al. 1982; Caul 1996).

In the early 1990s, the Norwalk virus genome was cloned and sequenced, establishing this virus as member of the family *Caliciviridae* (Green 2013). Later molecular studies allowed

classification of the virus into a new genus termed “Norwalk-like viruses” and more recently (2007) called genus *Norovirus* (Green 2013).

1.2. Virus taxonomy

Norovirus belongs to the *Caliciviridae* family, in reference to the cup-like (calyx) depressions on the virus surface (Green 2013). This family encompasses seven genera that infect mammals, *Norovirus, Sapovirus Vesivirus, Lagovirus, Nebovirus, Recovirus* and *Valovirus,* two genera infect birds, *Bavovirus* and *Nacovirus* and two fishes, *Minovirus* and *Salovirus*. Caliciviruses cause species-specific infection (Vinjé et al. 2019). The genus *Norovirus* is the most frequent cause of disease in humans, among the members of the *Caliciviridae* family (Vinjé et al. 2019). In this family, there are important animal pathogens namely the feline calicivirus, (genus *Vesivirus*) and the rabbit hemorrhagic disease virus (genus *Lagovirus*). Sapovirus mainly cause mild gastroenteritis in children up to 5 years of age, while norovirus is pathogenic for humans in all age groups (Svraka et al. 2010; Ahmed et al. 2014). In the next Table are the established genera, type virus and mammal hosts of the Caliciviridae family.

Table 1. *Caliciviridae* family classification including genus, type virus of mammal hosts

Genus	Type Species	Mammal Host
Norovirus	Norwalk virus	human, dog, swine, bovine, bat
Sapovirus	Sapporo virus	human, swine
Lagovirus	Rabbit hemorrhagic disease virus, European brown hare syndrome virus	rabbit, hare
Vesivirus	Vesicular exanthema of swine, virus Feline calicivirus, San Miguel sea lion virus	cat, swine, sea lion
Nebovirus	Newbury-1 virus	bovine
Recovirus	Tulane virus	simian
Valovirus	St Valerian	porcine

Adapted from Desselberger 2019.

1.3. Virus characteristics and life cycle

Noroviruses are single-strand RNA viruses of positive sense, non-enveloped with icosahedral symmetry nucleocapsid, with 27-37nm in diameter and have a 7.5-7.7kilobase genome (Green 2013). The genome serves as mRNA and as a template for a complementary negative strand being transcribed into genome RNA through the viral polymerase. The genome is organized in three open reading frames (ORF) (Figure 1). ORF1 encodes a 194-kDa polyprotein which is cleaved by the virus protease into six proteins, including the RNA-dependent RNA polymerase (RdRp) and other non-structural proteins responsible for transcription and replication. ORF2 encodes the major capsid protein VP1 a structural 64 k-Da protein, which forms the virus capsid and ORF3, considered the most variable region in the genome, encodes the minor capsid protein VP2 a 23-kDa protein that interacts with the genome RNA when the virion formation occurs. (Green 2013).

The VP1 protein is formed by the internal N-terminal shell (S) domain as well as a protruding (P) domain comprised of a P2 subdomain that is the most exposed part of the virus; the P1 subdomain lies below P2 (closer to the S domain). The viral capsid is formed by 180 copies (90 dimers) of VP1 symmetrically arranged (Pogan et al. 2018). The S domain surrounds the viral RNA, and the P domain, which is linked to the S domain through a flexible hinge, correspond to the C-terminal part of the VP1 protein. The highly variable P2 subdomain contains the putative neutralization sites and interacts with histoblood group antigens (HBGAs) (Vongpunsawad et al. 2013). VP2 protein is located inside the nucleocapsid and is most likely involved in capsid assembly and genome encapsidation (Vongpunsawad et al. 2013) VP2 is thought also to play a role in capsid stability and viral entry (Pogan et al. 2018; Gaziano et al. 2019).

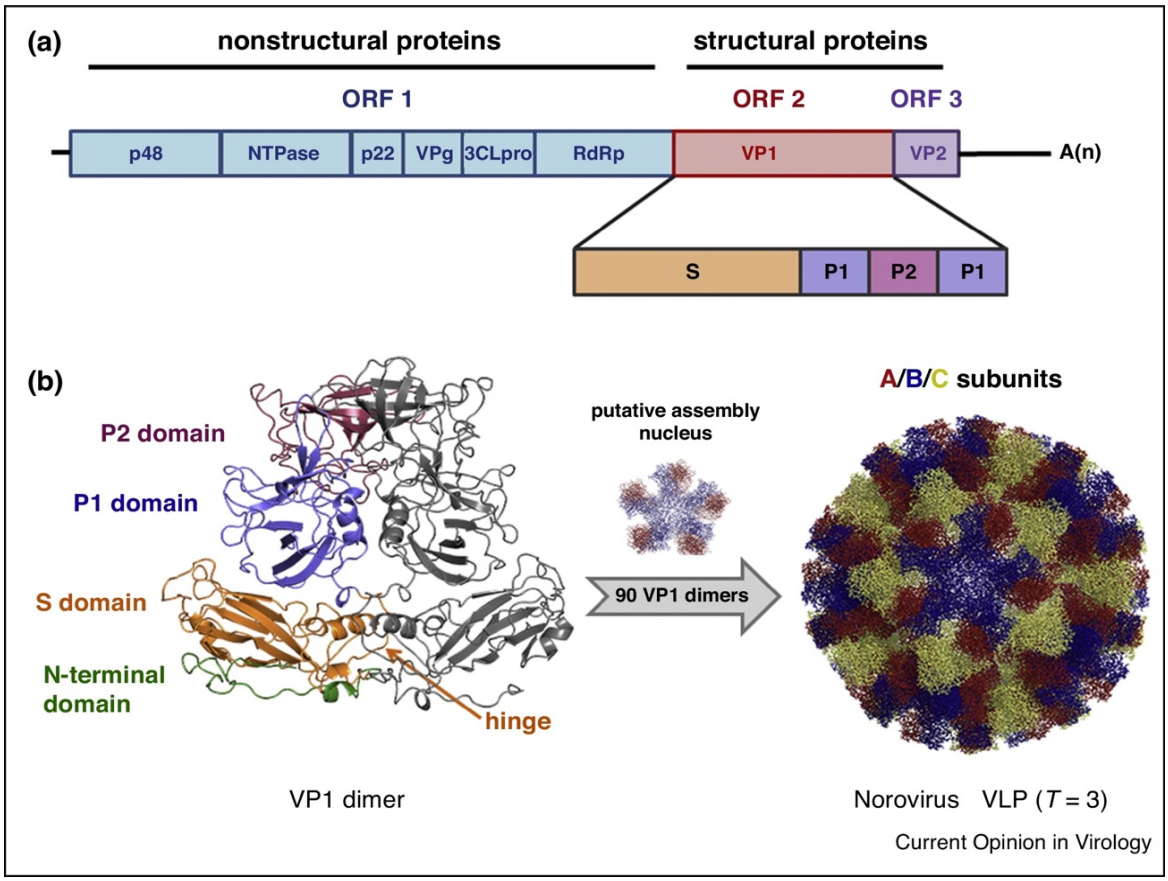

Figure 1. Human norovirus genomic organization and structure.

(a) norovirus have three ORF, ORF1, ORF 2 and ORF 3, respectively. A polyprotein which includes nonstructural proteins is encoded by ORF1. The two structural proteins, the major (VP1) and minor capsid protein (VP2), are encoded by ORF2 and ORF3, respectively. The VP1 protein is formed by the shell (S) and protruding (P) domains. (b) 90 dimers of the VP1 assemble into icosahedral T=3 norovirus like particles (VLP). The S domain of the VP1 monomers build a shell that surround the viral RNA in form of a scaffold. The more flexible P domain is subdivided into P1 and P2 and connect to S via a hinge. The domains are highlighted in the VP1 dimer structure (left) and the three quasiequivalent subunits (A/B/C) forming the capsids are shown in the VLP structure (Pogan et al. 2018).

Norovirus attach to host cells via a carbohydrate receptor of the histoblood group antigens (HBGAs) and probably another receptor entering into cells through clathrin and caveolin independent endocytosis (Green 2013; Ushijima et al. 2014). Bile salts and HBGAs are key mediators of norovirus entry; however, the molecular mechanisms by which these molecules promote infection and the identity of a potential human norovirus receptor remains unknown (Graziano et al. 2019). Inside the cell the virus is uncoated and the viral genomic RNA is released into the cytoplasm. The genomic RNA functions as a messenger RNA and it codes for the three ORFs. Translation is mediated by host translation factors that are recruited by non-structural protein VPg, which covalently binds to the 5´end of the genome (Green 2013; Ushijima et al. 2014). Viral genome-linked protein is then removed and viral RNA is translated into a processed ORF 1 polyprotein to yield the replication proteins. This polyprotein is cleaved

by the virus encoded protease (Pro) to produce six proteins: p48, nucleoside triphosphatase, p22, VPg, Pro and RdRp (RNA-dependent RNA polymerase). These proteins work in the replication process to copy the (+) sense genomic RNA into a (-) sense copy that is used as a template to produce a (+) sense sub genomic RNA and new genomic (+) sense RNA. The subgenomic RNA, which contains only ORF2 and ORF3, is translated to produce the major capsid protein (VP1) and a few molecules of a second capsid protein (VP2) that can self-assemble into virus like particles when these two proteins are expressed alone. At the end, new virus particles are assembled and release by cell lysis (Green 2013; Ushijima et al. 2014).

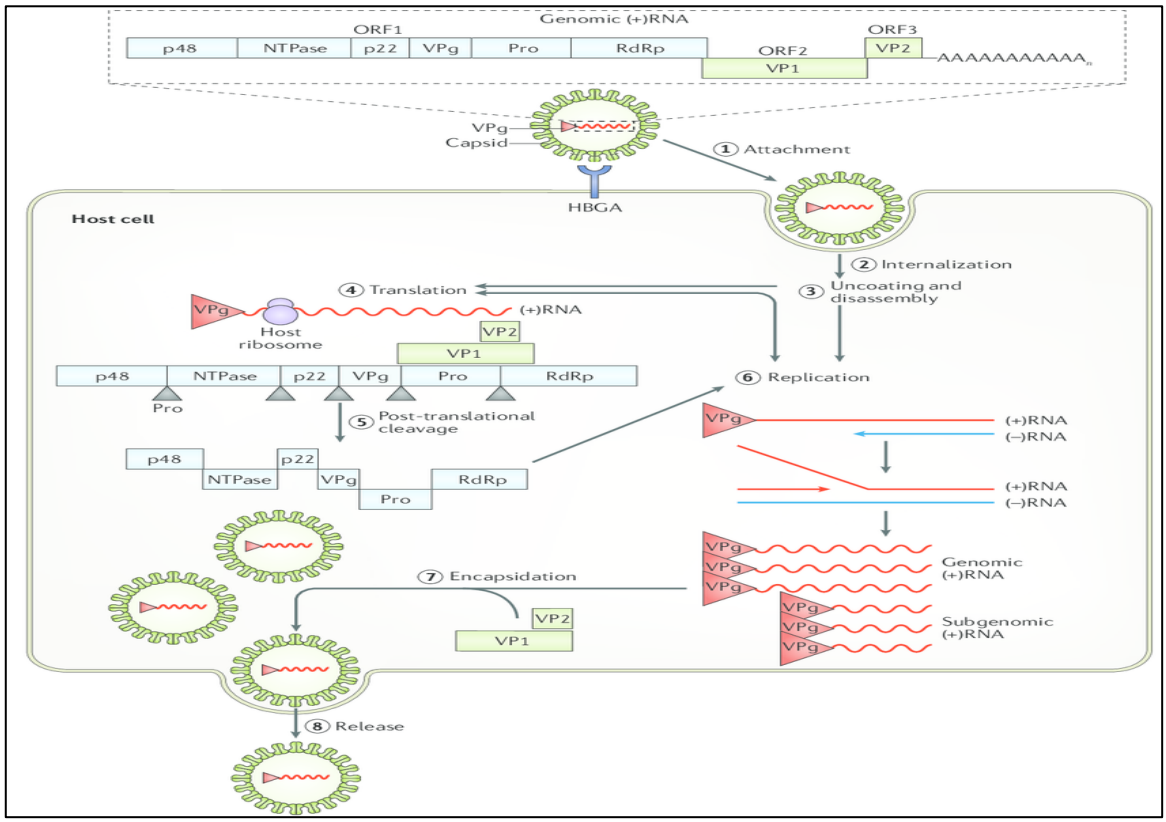

Figure 2. The composition and life cycle of human noroviruses (Graaf et al. 2016).

1.4. Genogroups and genotypes of norovirus

Noroviruses are genetically diverse viruses and are classified into different genogroups as well as polymerase (P) groups and further divided into genotypes and P-types based on aminoacid diversity of the complete VP1 gene and the nucleotide diversity of the RNA-dependent RNA polymerase region of ORF1, respectively (Chhabra et al. 2019).

Currently, noroviruses are divided in 10 genogroups (GI-GX) based on VP1 amino acid sequence diversity, in 49 genotypes (9 GI, 27 GII, 3 GIII, 2 GIV, 2 GV, 2 GVI and one genotype each for GVII, GVIII, GIX and GX), eight P-groups and 60 P-types based on partial nucleotide sequences of RdRp regions (Chhabra et al. 2019).

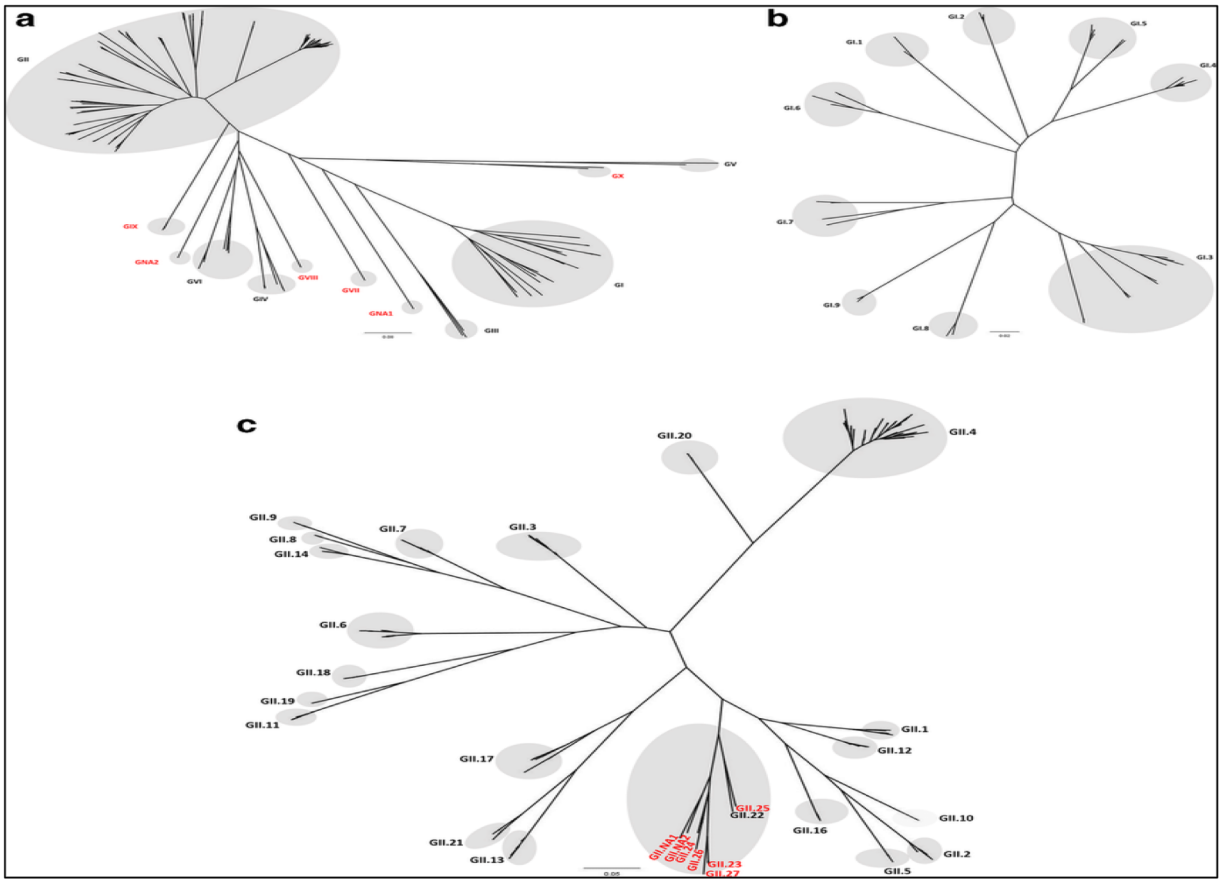

Figure 3. Genetic classification based on VP1 amino acid sequences into ten genogroups and two nonassigned genogroups (a), GI genotypes (b) and GII genotypes (Chhabra et al. 2019). The scale bar shows the number of nucleotide substitutions per site.

Norovirus within the same genogroup show 45-61% similarity in nucleotide sequence in the VP1 region and within the same genotype a similarity of 80 % (Zheng et al. 2006). New genotypes have been assigned when the VP1 amino acid sequence differs by more than 20% compared to other genotypes (Vinjé et al. 2000). In recent years, with the rapid accumulation of more norovirus sequence data, the cutoff threshold of 20 % needed adjustment due to increasing within-genotype diversity (Kroneman et al. 2013). So, within a genotype the amino acid divergence was changed to 14.1 % and a minimum of 15 % pairwise difference between the next-nearest genotype (Zheng et al. 2006).

Until 2019, a dual-nomenclature system was used, with the P-type designation linked to the corresponding VP1 genotype of the strain (Chhabra et al. 2019) and when no VP1 sequence was known, orphan P-types that only were seen in combination with established ORF2 genotypes, instead of a unique ORF2 genotype of their own, were designated by a letter, such as GII.Pc, GII.Pe and GII.Pg (Kroneman et al. 2013). As recombination in the ORF1-ORF2 junction region is common and some capsid genotypes seem to be more prone to recombination than others (Kroneman et al. 2013). A norovirus with a GII.4 polymerase and

capsid type were designated GII.P4-GII.4 and a recombinant GII.3 polymerase and a GII.4 capsid will be designated GII.3-GII.4 (Kroneman et al. 2013).

Classification and nomenclature updates in the future will be based on complete genome sequences (Chhabra et al. 2019).

1.5. Cultivation of human norovirus

Despite almost 50 years of attempts to develop *in vitro* culture methods this remains the major limitation to study human norovirus biology and the development of effective antiviral strategies (Estes et al. 2019). Routine cell cultures failed to yield replication of human noroviruses and attempts to develop a method for their cultivation were unsuccessful (Duizer et al. 2004). The noroviruses cell tropism has been an enigma for a long time, but it was assumed that human norovirus infected intestinal epithelial cells. Recent data support a more complex cell tropism of epithelial and nonepithelial cell types (Wobus 2018).The discovery of murine norovirus in 2003 and that it could replicate successfully in a murine macrophage line *in vitro* and primary immune cells *in vivo,* suggested that immune cells may also support replication of human norovirus (Wobus et al. 2004). Recently it was reported that a human B cell line supported the replication of a norovirus GII.4 strain in the presence of enteric bacteria (Jones et al. 2014), but its usage for in vitro replication of norovirus still awaits further developments (Jones et al. 2015). The human intestinal enteroids cultures reproduce the complexity and cell diversity of the gastrointestinal tract in relatively the same proportions as in the intestine itself (Zachos et al. 2016) and successfully support the replication of human norovirus (Costantini et al. 2018). These studies that confirmed enterocytes as the preferential site for human norovirus replication, support the genetic basis of host restriction and identify a role of bile as a strain-specific requisite or enhancer in virus infectivity (Ettayebi et al. 2016; Zou et al. 2017). Replication of human norovirus in Zebrafish and in organoids, made from pluripotent stem cells, has been recently reported enabling new studies on human norovirus biology (Van Dycke et al. 2019; Sato et al. 2019).

Successful cultivation was based on the discovery of genetically encoded host factors required for infection, knowledge of the site of infection in humans, and advances in the cultivation of human intestinal epithelium cells achieved by developmental and stem cells biologists (Estes et al. 2019).

1.6. Environmental resistance

Noroviruses are extremely stable in the environment, lasting weeks to years, depending on environmental conditions such as temperature and relative humidity (Seitz et al. 2011). They resist to nearly all of the active compounds of cleaning products, sanitizers, and disinfectants commonly used in food production and processing, including quaternary ammonium compounds, detergents, alcohols, and even chlorine at regulated concentrations (Hoezler et al. 2013). They can also survive to food processing and preservation methods such as heat, ionizing radiation, organic acids, preservatives, and manipulation of pH or water activity (Moore et al. 2015). Thus, human noroviruses are seen as the near-perfect foodborne pathogen, except for the fact that it cannot multiply (but does persist) in foods and the environment (Moore et al. 2015). Norovirus in various water sources are highly resistant to environmental degradation and long-term infectivity has been reported for groundwater which when seeded with the prototype norovirus (GI.1 Norwalk virus), in a clinical trial, was infectious for at least 61 days (Seitz et al. 2011).

Norovirus contamination of drinking water can be controlled by adequate free chlorine disinfection practices if proper pre-treatment processes are applied before chlorination (Shin and Sobsey 2008). In the environment, norovirus can be found in water that comes into contact with human stool samples which can also lead to contaminated crops (irrigation) and shellfish (growing waters) (Glass et al. 2009; Barclay et al. 2014; Katayama and Vinjé 2017). Norovirus also showed significant persistence in abiotic environments as it was reported that two carpet fitters who removed a carpet in a ward where a norovirus outbreak had occurred 3 weeks earlier, developed norovirus gastroenteritis symptoms (Cheesbrough et al. 1997).

In the presence of bacteria human norovirus was found to be more stable to acute heat stress, suggesting that bacteria may increase norovirus stability in the environment (Li et al. 2015; Graaf et al. 2017).

1.7. Epidemiology of norovirus infection

1.7.1. Disease impact worldwide

Norovirus is estimated to cause approximately 699 million illness cases and 219,000 deaths worldwide resulting in $4.2 billion in health system costs and $60.3 billion in societal costs annually (Bartsch et al. 2016). As most persons with acute gastroenteritis do not seek medical care and therefore do not incur healthcare costs, overlooking productivity losses would

severely underestimate the true cost of norovirus illness (Bartsch et al. 2016). Total cost estimates were most sensitive to hospitalization rates, probability of missing productive days, and care seeking rates (Bartsch et al., 2016).

Acute gastroenteritis causes the second greatest burden of all infectious diseases worldwide (Ahmed et al. 2014). Noroviruses are a leading cause of sporadic cases and outbreaks of acute gastroenteritis across all age groups, accounting for 18% of acute gastroenteritis cases worldwide (Ahmed et al. 2014). Norovirus prevalence, based on studies that used PCR based diagnosis, in patients with acute gastroenteritis, tends to be higher in cases in the community (24%) and outpatient settings (20%) compared with inpatient settings (17%) (Ahmed et al. 2014). Prevalence is also higher in low-mortality developing (19%) and developed countries (20%) compared with high-mortality developing countries (14%) (Ahmed et al. 2014). This may be explained by the fact that low-income countries have higher prevalence of other pathogens that cause acute gastroenteritis so the proportion of acute gastroenteritis caused by norovirus in these countries is relatively lower (Ahmed et al. 2014). Also, it should be noted in low-income countries medical services have lower coverage and the rate of underreported norovirus cases is probably higher (Nguyen et al. 2017). Another study estimates a norovirus prevalence of 17% in patients with acute gastroenteritis in developing countries (Nguyen et al., 2017). By age, prevalence was similar in patients with acute gastroenteritis under 5 years, 5 years and over, and of mixed ages (Ahmed et al. 2014; Nguyen et al. 2017). By country income, prevalence decreased as income decreased (Nguyen et al. 2017).

Although recognized as the leading cause of epidemic acute gastroenteritis across all age groups, norovirus has remained poorly characterized with respect to its endemic disease incidence. In the United States, norovirus causes an average of 570–800 deaths, 56,000–71,000 hospitalizations, 400,000 emergency department visits, 1.7–1.9 million outpatient visits, and 19–21 million total illnesses per year (Hall et al. 2013). People over 65 years of age are at greatest risk for norovirus-associated death while children under 5 years of age have the highest rates of norovirus associated medical care visits (Hall et al. 2013). In the US norovirus incidence is estimated at 700 illnesses/10,000 population, in the United Kingdom 450 illnesses/10,000 population, in the Netherlands 380 illnesses/10,000 population and in Canada 1,040 illnesses/10,000 population (Phillips et al. 2010; Tam et al. 2012; Hall et al. 2013; Verhoef et al. 2013).

Following the introduction of surveillance of outbreaks of gastrointestinal infection in England and Wales in 1992, norovirus was found responsible for more than 1800 outbreaks that affected more than 45,000 patients and hospital staff (Harris et al. 2014). Since 2009 another hospital-based surveillance system was implemented in England, the Hospital Norovirus Outbreak Reporting Scheme (HNORS) (https://www.gov.uk/government/publicat

ions/reported-norovirus-outbreaks-suspected-and-lab-confirmed-in-hospitals-2019). In the first 3 years (2009–2011) of the HNORS surveillance scheme, 4,000 outbreaks of norovirus were reported in England, affecting 40,000 patients and 10,000 staff (Harris 2016). Annually, on average these outbreaks affected 13 000 patients and 3400 staff, with 15 000 lost bed-days (Harris 2016).

In the aftermath of the hurricane Katrina a large norovirus outbreak affected more than 11,000 evacuees who were sheltered in the Reliant Park Complex in Houston, over an 11 day period (Yee et al. 2007). Future disaster preparedness and response planning should anticipate outbreaks caused by norovirus among evacuees, better prepared shelters with appropriate materials to educate about disease transmission and the importance of surveillance and prevention, and provide the proper policies, procedures, and resources to ensure good personal hygiene and sanitation for all involved (Yee et al. 2007).

In Portugal, information on prevalence of norovirus infection is scarce, but infections should occur frequently as antibodies against norovirus GII.4 were detected in 70% of a Portuguese cohort (Mesquita and Nascimento 2014). The underreporting of norovirus infections could be due to either the lack of knowledge among clinicians or limited availability of norovirus diagnostic testing in routine clinical laboratories (Mesquita and Nascimento 2014). In a two year hospital-based study performed in Portugal between 2011 and 2013, norovirus GII.4 (strain Sydney 2012) was detected in 11,6% of stool from patients hospitalized with acute diarrhea in 13 hospitals from Portugal (Costa et al. 2015).

1.7.2. Molecular epidemiology of norovirus

Noroviruses that infect humans have been associated only to genogroups GI, GII and GIV (Vinjé 2015). Noroviruses belonging to the genogroup GI and GII are responsible for the majority of disease cases in humans, whereas noroviruses from genogroup GIV are rarely detected as the cause of epidemic or sporadic gastroenteritis (Lindesmith et al. 2008; Robilotti et al. 2015). In humans, GII noroviruses are the predominant genotype detected (70%-89%), whereas noroviruses GI, which include virus of the GI.1 prototype Norwalk virus strain, cause approximately 11% of the outbreaks (Vinjé 2015; Lee et al. 2015). Within GII noroviruses genotype 4 (GII.4) are responsible for the majority of outbreaks and sporadic cases worldwide (Siebenga et al. 2009; Allen et al. 2016; Jung et al. 2017; Qin et al. 2017). In fact, GII.4 norovirus have caused all the six major pandemics of acute gastroenteritis in the last two decades (White 2014). These six pandemic GII.4 variants include US 96, which caused a pandemic in the late 1990s, Farmington Hills 2002, Hunter 2004, Den Haag 2006b, New

Orleans 2009 and Sydney 2012 (Leshem et al. 2013; Lee et al. 2015). These variants emerged in a periodicity of 2 or 3 years by genetic drift until 2012 (Van Beek et al. 2018).

An epidemiological study reported that in USA norovirus GII caused 72% of the outbreaks, 94% of each were either GII.4 New Orleans or GII.4 Sydney (Vega et al. 2014). The GII.4 Sydney seems to persist though recombination, as a norovirus recombinant of GII.p16-GII.4 Sydney 2012 variant was reported in Asia and Europe (van Beek et al. 2018). In China and Japan the genotype GII.4 was displaced by GII.17, which became the predominant genotype since 2014 (Qin et al. 2017).

Several non-GII.4 genotypes were significantly more associated with foodborne transmission and the cyclic emergence of new non-GII.4 norovirus strains and genotypes are also more often associated with foodborne outbreaks (Vega et al. 2014).

A study in Denmark investigated the genotype distribution in relation to age and setting and observed that norovirus GII.4 predominated in patients with 60 or more years and in health care centers while in children norovirus GII.P21 and GII.3 were more prevalent than in adults (Franck et al. 2014). It has been hypothesized that viral genotypes with lower antigenic variation, such as GII.3, more frequently infect children who do not have established immune-associated protection from previous norovirus infections (Franck et al. 2014). Moreover GII.4 strains are associated with more severe outcomes, including mortality, than infections during outbreaks of non-GII.4 strains (Desai et al. 2012).

1.7.3. Incidence seasonality of norovirus infection

Endemic norovirus disease occurs year-round but exhibits a pronounced winter peak and increases by around 50% during years in which pandemic strains emerge (Hall et al. 2013). In regions of temperate climate, norovirus occurrence is seasonal with most infections observed during the winter months (Katayama et al. 2008; Cannon et al. 2017). This annual fluctuation is likely caused by biological, environmental and behavioral factors that influence viral transmission, virulence and persistence in the host population (Rohayem 2009). Several studies have found associations between norovirus seasonality and climatic weather phenomena and specifically abundant rainfall and low temperatures seem to enhance viral persistence in water environments (Greer et al. 2009; Bruggink and Marshall 2010; Ahmed et al. 2014) The seasonality of norovirus gastroenteritis is also known to be influenced by the host behavior, in particular, crowding and prolonged sharing of indoors spaces are possible factors increasing human-to-human transmission of these viruses during winter (Mounts et al. 2000; Lindesmith et al. 2012; Zhou et al. 2016). As seasonal fluctuations modulate host cellular and humoral immune function, it is has been speculated that during wintertime diminished UV-radiation reduces vitamin D synthesis impairing immune response against

norovirus, as vitamin D is an important regulator of phagocyte function and is associated with the antiviral response to influenza virus infection by immune cells (Rohayem 2009).

In Europe norovirus seasonality coincides with northern hemisphere winter season, but GII.Pe/GII.P4-GII.4 strains show the clearest winter seasonal patterns while GI and other GII strains are more continuously present throughout the year (van Beek et al. 2018). China and Japan show increased norovirus incidence in the northern hemisphere winter season with the peak in November, two months earlier compared to Europe (van Beek et al. 2018). New Zealand shows highest incidence in October and November, and South Africa in September to November in southern hemisphere spring (van Beek et al., 2018). In the Middle east and North Africa countries peaks were observed during colder months, although norovirus infections were reported all year round (Kreidieh et al., 2017).

Climate change may affect norovirus seasonality with subsequent impact on norovirus transmission, host susceptibility to norovirus infection, resistance of norovirus to environmental conditions and change the interaction of norovirus with their host (Rohayem 2009). Human migration may become significantly altered as the result of climate change and as a consequence of floods or droughts, massive displacement of populations and crowding in refugee camps may facilitate the introduction of noroviruses into immunologically naïve populations, resulting in epidemics and the emergence of new norovirus strains (Rohayem 2009). In this context, norovirus evolution may be modulated by periods of elevated or facilitated transmission and evolutionary bottlenecks through rapid mutation or recombination events, which may in turn cause larger oscillations in the prevalence of the disease than are currently observed (Rohayem 2009).

1.7.4. Transmission of norovirus

Norovirus enter the human organism through the mouth. Virions are shed in large amounts in stools and in lower numbers in vomitus (Kilgore et al. 1996; Lee et al. 2007; Aoki et al. 2010). Virus expelling is highest during the acute phase of the disease, although shedding can last 9-56 days, even in asymptomatic persons (Atmar et al. 2008; Aoki et al. 2010). Peak shedding (10^{11} viral particles per gram of stool) occurs in the first days following infection but can persist for over three weeks, especially in young children (Atmar et al. 2008; Siebenga et al. 2008). In asymptomatic children shedding can last for 100 days and in immunocompromised individuals can be prolonged for several years (Murata et al. 2007; Ludwig et al. 2008; Schorn et al. 2010). Immunodeficient patients may become chronic symptomatic shedders although there is still no evidence that these individuals can transmit the virus and cause an infection (Koopmans 2005; van Beek et al. 2017). In human challenges studies approximately 30% of norovirus GI (GI.1) infected individuals are asymptomatic (Atmar

et al. 2008; Kirby et al. 2014). Infected people who are asymptomatic may shed similar amounts of virus as ill persons (Koopmans 2005; Newman et al. 2016).

Patients shedding low levels of norovirus RNA may not be infectious, as a recent study seemed to demonstrate with norovirus GII.pe-GII.4 Sydney in a human intestinal enteroid model (Chan et al. 2019).

Norovirus can be transmitted by different ways such as through food, persons, water, and even the environment (Verhoef et al. 2015). The most important mode of transmission is the fecal-oral spread although infectious vomit, either by mechanical transmission from environmental surfaces (hand/mouth contact) or by aerosolization might account for the rapid and extensive spread of disease outbreaks, especially in closed settings (Patel et al. 2009).

Norovirus are infectious by oral route at very low doses (Glass et al. 2009), with an, estimated dose of 18 to 1000 of viral particles (Teunis et al. 2008).

Sometimes it is difficult to determine the transmission of norovirus, as more than a route can occur in a single outbreak. After primary introduction of the norovirus through food, secondary person-to-person and environmental transmission can rapidly take over, making it hard to trace the disease back to contaminated food. Another complexity is that foodborne transmission can follow different routes as well (Verhoef et al. 2015).

Person-to-person transmission is thought to account for 62–84% of all reported outbreaks (Moore et al. 2015). A study of norovirus outbreaks in Europe revealed that primary transmission occurs through the fecal-oral route, by person-to-person spread in 88%, ingestion of norovirus contaminated food in 10% or water in 2% (Kroneman et al. 2008).

Using data from the CaliciNet surveillance system from 2009 to 2013 in USA the estimated transmission in norovirus outbreaks was 83.7% for person-to-person and 16.1% for foodborne (Vega et al. 2014). In the same study, 62.5% of the outbreaks occurred in long-term care facilities, 9.8% in restaurants and 5.8% in schools. Applying the profiles in surveillance database, the proportion of outbreaks attributed to foodborne transmission varied slightly with a global estimate of 13.7% (Verhoef et al. 2015). More recently in another European study, 77,4% of the transmission was considered person-to-person, 19,9% foodborne, 2,1% waterborne and 0,7% from other modes of transmission (van Beek et al. 2018).

The epidemiologic curves are usually distinct between outbreaks involving person-to-person or food-borne transmission. In the person-to-person transmission the numbers of patients increase and then decrease gradually, on the other hand in the foodborne transmission, infection occur at once and intensively, since the cohort is mostly exposed to the contaminated food at the same time (Ushijima et al. 2014). Norovirus GII.4 is relatively more often associated to person-to-person transmission than other genotypes (van Beek et al. 2018).

Animal noroviruses has not yet been found in humans, however detection of human noroviruses in animals and simultaneous animal and human viruses in bivalve mollusks suggests a risk of transmission (Bank-Wolf et al. 2010). Human noroviruses have been detected in stool of dogs, swine, cattle, wild birds and rodents (Mattison et al. 2007; Caddy et al. 2015; Summa et al. 2018). Canine seroprevalence to different human norovirus genotypes resembles the seroprevalence in the human population (Caddy et al. 2015). Serum antibodies against bovine and canine noroviruses have also been detected in humans, with higher level in veterinarians than in the general population (Widdowson, Rockx, et al. 2005; Mesquita et al. 2013). A recent study found more evidence for human noroviruses in animals than the reverse, suggesting that human noroviruses could be a reverse zoonosis, although it is still too early to consider norovirus a zoonotic or reverse zoonotic pathogen (Villabruna et al. 2019). Zoonotic transmission was never proved. Pet dogs and wildlife (birds and rodents) may play a role in the spreading of human noroviruses in the environment (Summa et al. 2012; Summa et al. 2018).

1.7.5. Norovirus and foodborne and waterborne illness

Norovirus is the leading cause of acute gastroenteritis and foodborne disease outbreaks in the United States of America (USA) (Hall et al. 2014). It is estimated that in the USA viruses account for 59% of the foodborne diseases and that norovirus account for 99% (5.5 million) of all viral foodborne illness incidents per year (Scallan et al. 2011) . Norovirus is also responsible for 26% of hospitalization and 11% of death related to foodborne diseases (Scallan et al. 2011). In Canada, norovirus is also considered the leading cause of foodborne illness, accounting for 65% of known illnesses (Thomas et al. 2013). In Australia norovirus is the leading cause of foodborne illness, accounting for 30% of illnesses caused by known pathogens (Hall et al. 2005). In England and Wales, norovirus accounted for only 8% of known foodborne illnesses (Scallan et al. 2011). Foodborne illness of norovirus origin by country or region is summarized in Table 2.

As reported by the European Food Safety Authority (EFSA), in 2017, in the European Union (EU) viruses accounted overall for the 9.8% of total foodborne and waterborne outbreaks in 2016, which is comparable with 2015. At the EU-level, no trends in the outbreak number reported were observed for calicivirus (including norovirus). During the 2010–2016 period, Denmark, Lithuania and Sweden reported a statistically significant decreasing trend in the number of outbreaks of calicivirus including norovirus, while France and the Netherlands reported a statistically significant increase. Three other European countries (Finland, Germany

and United Kingdom) reported a 2016 increase in the number of outbreaks by calicivirus including norovirus of over 50% as compared with the previous year (EFSA 2018).

Table 2. Foodborne illness of norovirus origin

Country or Region	Foodborne illness of norovirus origin (%)	References
USA	59	Hall et al. 2014
Canada	65	Thomas et al. 2013
Australia	30	Hall et al. 2005
England and Wales	8	Scallan et al. 2011
EU	9.8	EFSA 2018

Foodborne norovirus outbreaks are difficult to identify and even more difficult to trace back to a common food source as most foodborne norovirus outbreaks are small, associated with a retail setting (and hence investigated locally or regionally) and often with secondary person-to-person spread (propagated outbreaks) (Moore et al. 2015). Contamination of food frequently happens at the end of the production-logistic chain with typical examples of a food handler with poor hand hygiene or persons who were sick inside a room where food was prepared or stored (Koopmans 2005). Larger foodborne outbreaks affecting vast geographic regions have also been detected (Koopmans and Duizer 2004; Koopmans 2005; Bernard et al. 2014; Morgan et al. 2019). In this case it is thought that the contamination event is early in the food chain, for instance with berries that are harvested under poor hygienic conditions, or that were irrigated or washed with contaminated water (Koopmans and Duizer 2004; Koopmans 2005).

The majority of norovirus outbreaks were due to person-to-person transmission, although foodborne transmission is also significant (Moore et al. 2015). Complex, prepared, ready-to-eat foods are overrepresented in outbreaks, and the most common settings for these are restaurants, delicatessens and catering facilities, implicating food handling as the likely contamination source (Moore et al. 2015). In the case of simple foods, vegetables, fruits, and nuts are the most likely culprits, but it is difficult to discern how relevant was pre-harvest contamination in these products (Moore et al. 2015).

In a study performed in USA foodborne transmission was observed in 23% norovirus outbreaks during the 4 year study period, while person-to-person transmission accounted for 69%, environmental for 0,35%, waterborne for 0,26% and unknown transmission for 7% (Figure 3) (Hall et al. 2014). In a study, in China, from 2014 to 2017, the majority of the

outbreaks (63%) were due to person-to-person transmission, followed by multiple modes of transmission (11%), foodborne (5%) and waterborne (3%) transmission (Lian et al. 2019).

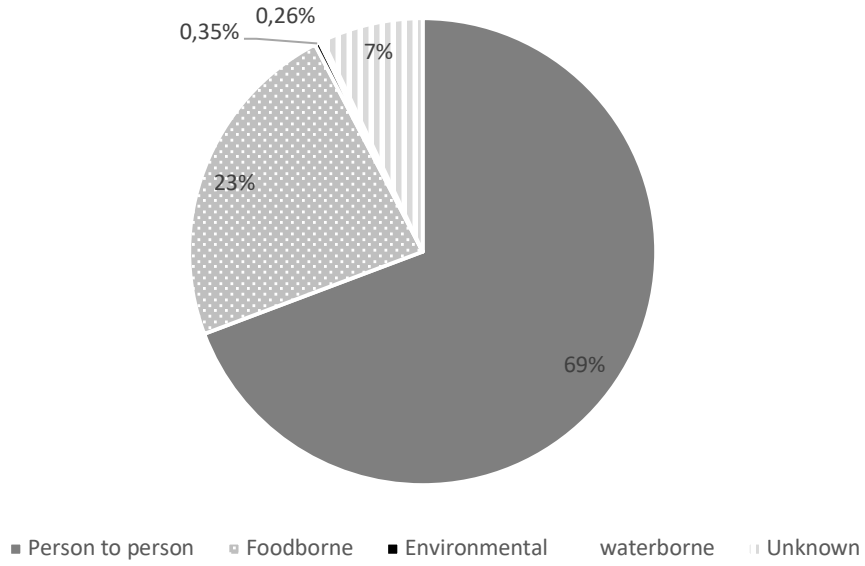

Figure 4. Transmission routes of norovirus in the USA (adapted from Hall et al. 2014).

Of the norovirus outbreaks that could be attributed to a single location, restaurants and delicatessens were the most common (63–64%), followed by catering and banquet halls (11–17%) and private homes (4–6%) (Hall et al. 2014). Food workers were implicated as the source in 70% of the outbreaks with an identified cause. Most outbreaks with a demonstrated implicated food resulted from food contamination during preparation (92%) and food consumed raw (75%). The most frequently food categories implicated in the outbreaks were vegetable row crops (e.g., leafy vegetables) (30%), fruits (21%), and mollusks (19%) (Hall et al. 2014).

In the EU according to EFSA report of 2017, zoonotic agents and food-borne outbreaks in 2016, caused by norovirus ranked first among the causative agents of outbreaks caused by ‘fish and fishery products’ (51.4%) and was also reported in high proportions in outbreaks by ‘other foods’ (22.8%), ‘mixed food and buffet meals’ (22.3%) and ‘vegetables, fruits, cereals, sprouted seeds, herbs and spices and their products’ (26.4%) (EFSA 2017).

In another study on a global scale it was estimated that 14% of norovirus outbreaks were attributed to food (Verhoef et al. 2015). The proportion of norovirus outbreaks attributed to foodborne transmission is in the same order of magnitude as the 17% found in a study from the Netherlands and 11% in the United Kingdom as estimated from outbreak surveillance data (Adak et al. 2002; Havelaar et al. 2012; Verhoef et al. 2015). Interventions to reduce the frequency of foodborne norovirus outbreaks should focus on food workers, food production and shellfish as most foods are likely contaminated during preparation and service, except for

mollusks, and occasionally contaminated during production and processing (Hall, Eisenbart, et al. 2012).

The number of foodborne illnesses caused by enteric bacteria have in generally declined over the past two decades (Center for Disease Control and Prevention [CDC] 2006) and possible reasons for this trend include better public awareness of safe cooking and hygiene food handling practices, improved standards in industrial food processing and better refrigeration and storage (Widdowson, Sulka, et al. 2005). On the other hand, the number of foodborne outbreaks caused by norovirus have remained unchanged or have increased and were estimated at 30-50% of all foodborne outbreaks (CDC 2006). It is likely that control measures in the food industry and public health awareness campaigns aimed at reducing foodborne bacterial illness have done little to reduce norovirus gastroenteritis (Cannon 2008).

Norovirus genotype profiles can be used to differentiate foodborne outbreaks caused by food contamination early in the food chain from those caused by food handlers contaminating food (Verhoef et al. 2010). In a study in Denmark, norovirus GI was more frequently observed in food-borne outbreaks than in outbreaks involving person-to-person transmission and was also more frequent in foodborne outbreaks than in outbreaks in health care and community settings (Franck et al. 2014). In outbreaks in community settings, GII infections outnumber GI infections, as GII strains have been found to be associated to 80% or more of outbreaks with the epidemic strains belonging to GII.4 being responsible for the vast majority of cases, particularly those associated with person-to-person transmission (Matthews et al. 2012; Vega et al. 2014). In the USA, 72% of the outbreaks reported from 2009 to 2013, were caused by GII.4 strains and non-GII.4 strains were significantly more associated with foodborne transmission (Vega et al. 2014).

Norviruses strains detected in foodborne outbreaks showed a genotype profile similar to those in bivalve mollusk monitoring (predominance of GI strains) and dissimilar to the profile detected in human stools with respect to the frequently seen genotypes which could reflect the ability of these genotypes to survive outside humans or their diminished ability to spread or replicate within the human population (Verhoef et al. 2010).

The genotype profiles and proportions may be helpful for estimating the number of outbreaks with potential of geographic dissemination. Early detection should enable containment of viral foodborne infection and thus prevent further spread to large numbers of human infections as the identification and investigation of such outbreaks provides insight into effective prevention measures during the production process (Verhoef et al. 2010).

1.8. Human norovirus infection

1.8.1. Pathogenesis of human norovirus infection

Norovirus recognize HBGA in a strain-specific manner (Chen et al. 2011). All three major families of HBGA, the AB0, Lewis and secretor families are involved in binding noroviruses (Huang et al. 2003; Tan and Jiang 2005). Persons carrying a functional fucosyltransferase 2 (encoded by FUT2 gene) are termed secretors and express HBGAs, whereas homozygous individuals, called non- secretors, are almost completely protected from GI.1 and GII.4 norovirus infections (Jin et al. 2013). However, polymorphisms in the FUT2 genes vary considerably depending on ethnicity and non-secretors can be infected by other norovirus genotypes (Jin et al. 2013). Because GII.4 viruses can bind a wider range of HBGAs than other genotypes, they are able to infect a larger susceptible population (Vinjé 2015).

After entering the gut lumen noroviruses gain access to inflammatory cells through microfold cells (M cells),a specialized subset of enterocytes that overlie Peyer´s patches and isolated lymphoid follicles and are highly efficient at sampling and transporting luminal material to the underlying immune aggregates (Karst and Tibbetts 2016). Noroviruses can infect both innate (macrophages and dendritic cells) and adaptive (B cells) immune cells along the intestinal tract, and infection of these cell types is a crucial determinant of viral pathogenesis (Karst and Tibbetts 2016). It seems that commensal bacteria enhance norovirus infection and facilitate infection persistence by direct mechanisms like the expression of H type HBGAs that stimulate norovirus adherence to permissive B cells and also by indirect mechanisms in the form of immunomodulation (suppression of type III IFN for example) (Karst and Tibbetts 2016).

The intestinal mucosa appears to remain intact during human norovirus infection, but there are histopathological changes in the epithelium, like the broadening and blunting of the villi (Karst 2010). Crypt cell hyperplasia has also been reported following norovirus infection (Karst 2010). It is thought that these changes can impair the absorption of D-xylose, fat and lactose and the changes of secretory and/or absorptive processes are thought to be in the origin of the diarrhea (Karst et al. 2015). The pathophysiology of the vomit is thought to be related to a marked delay in gastric emptying, possible due to abnormal gastric motor function (Karst et al. 2015) .

1.8.2. Norovirus gastroenteritis

Typically, the incubation period of norovirus gastroenteritis is 12-48 hours (Katayama and Vinjé 2017), although it can vary from 10 to 51 hours (Glass et al. 2009).

Viral gastroenteritis is generally a mild and self-limiting illness but patients sometimes feel devastating illness for 24 to 48 hours (Glass et al. 2009; Robilotti et al. 2015). Severe disease is seen in risk groups and the most vulnerable are the elderly, young children, the immunocompromised. and people with underlying illness (Koopmans 2005; Bok and Green 2012; Vega et al. 2014; Cardemil et al. 2017). The severity is different in individuals depending on the viral infecting dose and host factors such as the immunity status (Ushijima et al. 2014).

The disease often begins with vomiting (in more than 50% of patients) and nausea, followed by abdominal cramps, fever (in 37 to 45% of the cases), non-bloody watery diarrhea, and other symptoms such as headache, chills, and myalgia (Arness et al. 2000; Glass et al. 2009; Robilotti et al. 2015). Usually the patients recover within one to three days (Robilotti et al. 2015). Complications of convulsion, encephalopathy, necrotizing enterocolitis and nephropathy have also been reported in some cases (Ito et al. 2006; Kanai et al. 2010; Ushijima et al. 2014).

Older adults are particularly at increased risk of severe outcomes, including prolonged symptoms and death (Mattner et al. 2006; Hall, Curns, et al. 2012). Advanced age is a risk factor for a fatal outcome (Robilotti et al. 2015). Long term care facilities and hospitals are the most commonly reported settings for norovirus outbreaks in developed countries, and older adults (>65 years of age) in these settings are more likely to experience health care associated infection with more severe infections and poor outcomes (Lopman et al 2004; Franck et al. 2014; Vega et al. 2014; Cardemil et al. 2017). Longer hospital stays, increased exposure and susceptibility to the virus due to age-related changes in B-cell and T-cell function and immunosenescence or underlying chronic conditions and comorbidities could explain these observations (Cardemil et al. 2017).

Infants and young children can experience more prolonged infections that can last up to 6 weeks (Murata et al. 2007; Desai et al. 2012). Young children are also at risk (Robilotti et al. 2015). A study in the USA demonstrated that children under 5 years have the highest rates of norovirus associated health care visits (Hall et al. 2013). Amongst norovirus infected children in Africa, HIV-infection was associated with prolonged hospitalization and increased mortality (Page et al. 2017).

Immunosuppressed persons due to congenital or acquired immunodeficiencies, transplant, receipt of immunosuppressive therapy and cancer are at increased risk for prolonged and more severe norovirus illness (Mattner et al. 2006; Bok and Green 2012).

Underlying conditions as cardiovascular disease or renal transplant may lead to severe consequences typified by decreased potassium levels, increased levels of C-reactive protein and creatine phosphokinase in patients with norovirus infections (Mattner et al. 2006).

Severe dehydration occurs in high-risk individuals, such as infants, the elderly, and immunocompromised hosts (Glass et al. 2009; Green 2013). More severe illness may,

however, occasionally occur in previously medically healthy individuals (Robilotti et al. 2015). In military personnel both in training or war like conditions, thrombocytopenia, photophobia, disseminated intravascular coagulation and the need of ventilatory support have been reported (Arness et al. 2000; Ahmad 2002).

There is also evidence that norovirus has a role in travelers' diarrhea (Karst et al. 2015). Studies have shown prevalence rates of noroviruses in travelers' diarrhea cases ranging from 10–65% (Simons et al. 2016).

1.8.3. Immunologic response to norovirus

Evidence for both short-term and long-term immunity immune responses has been demonstrated, but the mechanisms mediating differential immune responses in the face of norovirus infection remains unclear (Donaldson et al. 2008). Typically, immunity is strain- or genotype specific, not life-long and with an estimated immunity duration from six months to eight years (Simmons et al. 2013; Lopman et al. 2016). Adult can be recurrently infected by norovirus due to either short persistence of protective antibodies or inefficacy of antibody cross-protection against distinct genotypes (Ushijima et al. 2014). Within genogroups there is some cross-protection following infection, more evident for GI than GII strains but between genogroups cross protection is minimal or absent (Debbink et al. 2012; Sakon et al. 2015).

Seroprevalence studies suggest that humans are frequently exposed to noroviruses, with seropositivity rates reaching more than 90% worldwide (Pringle et al. 2015; Robilotti et al. 2015; Melhem 2016). In Portugal, viral antibodies to GII.4 norovirus were detected in 70% of adults (Mesquita and Nascimento 2014).

Consistent with the short duration of norovirus symptoms, innate immunity responses, specially type I Interferons (IFNs), are critical for controlling acute norovirus infections although components of the adaptive immune response also contribute to the control and clearance of primary norovirus infections (Karst et al. 2014). Norovirus infection induces a systemic immune signature in the innate and adaptive immune systems, transiently increasing serum IFNγ, Interleukin-2 (IL-2), IL-6 and tumor necrosis factor-α (TNFα) with counter-regulatory mechanisms (production of IL-10) instructed early in the response to infection, to temper collateral tissue damage (Cutler et al. 2017).

Norovirus induce mucosal and serum antibody responses and studies in mouse model confirms that B cells and antivirus antibodies are critical to protect from secondary norovirus infections (Karst et al. 2015). The presence of the virus-specific mucosal and serum IgA correlates with protection of infection suggesting that the CD4+ and CD8+ T cell responses

are involved (Karst et al. 2015). Serum antibody that blocks the binding of norovirus to HBGAs is the best studied and the leading correlate of protection (Lopman et al. 2016).

1.8.4. Immune evasion and lack of vaccine against norovirus

The persistence of norovirus in human population may be fueled by virus mutation, through two different mechanisms: different receptor usage and different antigenic structure (Donaldson et al. 2008). Immunity against norovirus seems to persist for four to eight years (Simmons et al. 2013) supporting the notion that anti norovirus herd immunity exists (Sakon et al. 2015). Genetic variation of antigens allows escape from the predominant herd immunity resulting in a virus competent to infect the same population that has previously been infected (Donaldson et al. 2008; Debbink et al. 2012). Dynamic antigenic mutations are probably an important trigger of norovirus endemics (Sakon et al. 2015).

The majority of norovirus outbreaks are caused by GII.4 genotype, with new pandemic strains arising every two to three years. Within this particular genotype, herd immunity is the driving force of evolution (Karst et al. 2015). Antigenic drift, strain recombination, antigenic shift and reduced polymerase fidelity of norovirus clearly contribute to the continuous propagation of GII.4 genotype (Melhem 2016). The continued prominence of GII.4 strains has been explained by evolution within the P2 subdomain of these strains that leads to receptor switching and antigenic drift, resulting in both access to previously resistant populations and evasion of protective immunity in previously susceptible populations (Lindesmith et al. 2010).

The GII.4 noroviruses, and to a lesser extent one variant of the GII.17 viruses, acquired amino acid substitutions over time that created phenotypically different variants. In contrast, all other genotypes retained similar sequences within variants that might have arisen early in the origin of that genotype and that persisted over time (Parra et al. 2017). Two different patterns of evolution in norovirus seems to occur: evolving and static. Evolving viruses continually accumulate mutations in their genome over time, and static viruses do not (Parra et al. 2017).

No clinical vaccine is available to prevent norovirus illness and infection (Ong 2013). The development of an effective vaccine has been hindered by the extreme diversity of norovirus and by its uncultivable nature (Karst et al. 2014). Clinical trials of vaccines consisting of non-replicating virus-like particles have shown promise (Karst et al. 2014). Human clinical trials have proved to date that norovirus virus-like particles vaccines are safe and immunogenic, although a number of factors hamper the generation of an efficient and protective vaccine against norovirus. The incomplete understanding of the virus shedding dynamics and its heterogeneity, the complications of diversity, evolution and selective pressure

of the virus, and the debatable estimated immunity contribute to delaying the development of a successful vaccine (Melhem 2016).

1.9. Laboratory detection of norovirus

Due to the inability of human norovirus to grow in cell culture the first and the only method used for their detection, until the implementation of the molecular techniques, was electron microscopy (Vinjé 2015). The low sensitivity of this method and the lack of accessibility of the majority of microbiology laboratories to electron microscopy hampered the diagnostic of norovirus infections that was based during a long time on clinical and epidemiological characteristics (Vinjé 2015) that differentiate them from the other infectious gastroenteritis. These characteristics are known as the Kaplan criteria: a mean illness duration of between 12 and 60 h, a mean incubation period of 24 to 48 hours, vomiting in at least 50% of patients and the absence of bacterial pathogens identified in stool samples (Kaplan et al. 1982). In food borne outbreaks this criteria have a specificity of 99% and a sensitivity of 68% (Vinjé 2015). The preferred clinical specimen for the diagnostic of norovirus infection are whole-stool samples although rectal swabs and vomitus could also be used (Ong 2013; Vinjé 2015).

1.9.1. Molecular methods to detect norovirus

Molecular assays were possible after the Norwalk virus genome cloning and characterization that allowed the development of sensitive diagnostic assays (Xi et al. 1990). The first Reverse-Transcription-PCR assays (RT-PCR) were described within 2 years of the initial report of the successful cloning of norovirus genome (Jiang et al. 1992). The first molecular assays developed to detect noroviruses were conventional RT-PCR assays targeting a conserved small region of the RdRp (POL) gene in ORF1 (region A) (Figure 4) (Vennema et al. 2002). With the increasing number of norovirus genome sequences that became available, those assays were replaced by second-generation assays that proved to be more broadly reactive and able to detect the majority of the circulating norovirus strains (Vinjé 2015).

The use of Reverse-Transcription-quantitative PCR (RT-qPCR) highly increased the specificity and sensitivity of norovirus detection that together with the advantages of one-step RT-qPCR reaction (both reverse transcription and cDNA amplification are performed in a single reaction decreasing the risk of cross-contamination) making them a preferred format in clinical laboratories (Vinjé 2015). The gold standard method for the rapid and sensitive

detection of noroviruses in clinical as well in food, water and environmental samples is RT-qPCR targeting ORF1-ORF2 junction (region C) (Figure 4) as this is the only sufficiently conserved region (Vega et al. 2011; Vinjé 2015).

Molecular assays cannot distinguish between infectious and non-infectious viruses, although they are very specific and have high analytical sensitivity (Chan et al. 2019).

Recently several commercial multiplex molecular assays have been developed for the simultaneous detection of pathogenic enteric viruses, bacteria and parasites. These tests varied greatly in type of detected microorganisms, number of processed samples, duration and workflow. Caution must be taken when interpreting the tests output, especially in cases of high numbers of mixed infections and lack of quantitative data to determine which pathogen is responsible for the outbreak (Vinjé 2015).

Nucleotide sequencing of RT-PCR products has been particularly useful in molecular epidemiology studies to identify point-source of infection, as well as to differentiate outbreaks that were mistakenly assumed to be connected (Patel et al. 2009).

Sequencing of the complete VP1 gene is not currently a routine procedure, so for genotyping small regions of ORF1 (region A) or ORF2 (regions C and D) of the virus genome are most commonly used (Vinjé 2015). Region C assays are often more robust because the lower annealing temperature required for region D assays increases the likelihood of nonspecific amplification and because region D is located in a more variable part of ORF2 (Kroneman et al. 2011; Vinjé 2015). An online norovirus typing tool, developed by the National Institute for Public Health and the Environment of the Netherlands, is available for both polymerase (Region A and B) and capsid typing (Region C, D, E) (Figure 4) and was designed to use phylogenetic methods in order to identify the norovirus of a nucleotide sequence (https://www.rivm.nl/mpf/typingtool/norovirus/).

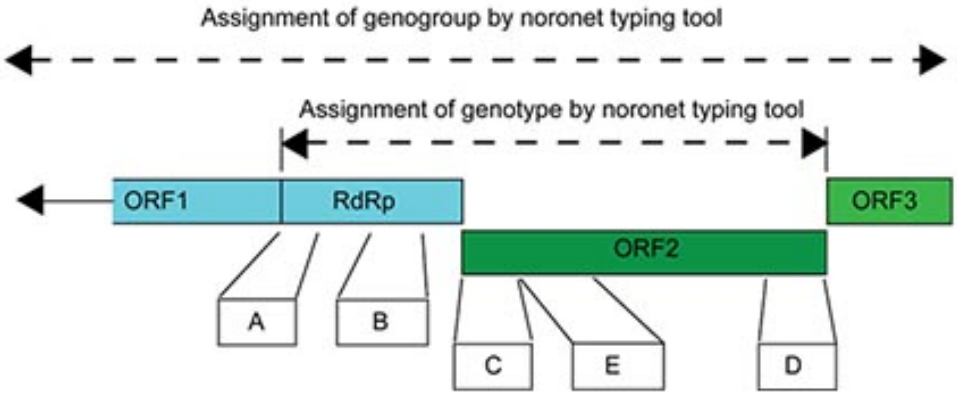

Figure 5. Genome regions used for genotyping of noroviruses (http://www.rivm.nl/mpf/norovirus/typingtool).

Next generation sequencing (NSG) is starting to be used in investigation of norovirus outbreaks and novel variants, in the detection of epidemic genotypes in raw sewage or in the analysis of the diversity of human noroviruses (Fonager et al. 2017; Imamura et al. 2017; Fumian et al. 2019). An important application of NGS for clinical and public health work is the ability to track outbreaks, identify sources of introduction and track transmission in a care facility or hospital to the level of individual transmission events (who infects whom) (Cotten and Koopmans 2016).

The molecular tests allow the collection of stools using Whatman™ "FTA" papers to collect and transport the samples to reference laboratories without the use of storage conditions of 4ºC or -20ºC (Delacour et al. 2010). This procedure allows the maintenance of the samples at room temperature for eleven weeks before the molecular testing (Delacour et al. 2009).

1.9.2. Immunological methods

A rapid detection of norovirus is important for the implementation of effective measures to control an outbreak. Rapid tests were developed for the detection of norovirus antigen in stool samples such as immunochromatographic tests that gives an answer in 15 minutes and does not require specialized laboratory equipment (Vinjé 2015). These tests usually have a 100% specificity but a poor and variable overall sensitivity dependent on the genogroup and genotype, ranging from 35% to 52% for norovirus GI and variants from 59% to 78% for norovirus GII.4 (Ambert-Balay and Pothier 2013).

The development of enzyme immunoassays (EIAs) for detection of norovirus antigen using fecal specimens has been hampered by the antigenically diversity of human norovirus genotypes and the antigenic drift of certain strains (Vinjé 2015). Nevertheless, some commercial kits are available such as IDEIA™ Norovirus EIA (Oxoid Hampshire, United Kingdom) and RIDASCREEN Norovirus (r-Biopharm AG, Darmstadt, Germany). The sensitivity of EIA is usually 70% and their specificity about 90%, depending on the diagnostic goal (outbreak or sporadic cases), the number of samples tested per outbreak, and the time after the onset of symptoms that clinical samples were collected (Vinjé 2015). In fact, the sensitivity for detecting norovirus in fecal samples from outbreaks improved from 44.1% when three samples were tested to 76.9% when the number of samples tested per outbreak increased from three to five (Costantini et al. 2010). Norovirus antigen EIAs had showed low clinical sensitivities, from 11% to 35%, for specific genotypes, such as GII.17 Kawasaki 2014 variant (Chan et al. 2016). False negative results can compromise infection control and patient management (Chan et al. 2016). Older studies had also reported different sensitivities in different genotypes

of genogroup II, stating that the reactivities of GII.3 and GII.6 should be improved (Okitsu-Negishi et al. 2006).

The current EIAs may be of use as a rapid screening test during a norovirus outbreak investigation when multiple fecal samples are available, however, sporadic samples should be tested by molecular methods (Costantini et al. 2010, Ambert-Balay and Pothier 2013).

1.9.3. Detection of norovirus in environmental and food samples

Despite the advances in laboratory techniques, the detection of noroviruses in environmental and food samples is often unsuccessful (Rutjes et al. 2006). This can be explained by the low numbers of viral particles in the environment or contaminated foods (Moore et al. 2015). Until de mid-2000s, sample concentrates were processed for RNA isolation followed by detection by conventional RT-PCR and confirmation of amplicon using nucleic acid hybridization or sequencing (Moore et al. 2015). In the middle of the last decade, RT-PCR was replaced almost exclusively by RT-qPCR, bypassing the time-consuming DNA hybridization and sequencing steps. This method currently remains the gold standard (Vinjé 2015).

Therefore, the viruses must be concentrated and purified from complex food matrices prior to detection with molecular amplification which made the detection laborious and time consuming. There are innumerous procedures described in the literature for the detection of norovirus from foods, water and the environment and a standard was released by the European Committee for Standardization (CEN) and the International Organization for Standardization (ISO) in 2013 - the ISO 15216:2013 (https://www.iso.org/standard/55382.html). This standard which consisted of two parts, describes a method for detection and quantification of levels of hepatitis A virus and norovirus genogroup I (GI) and II (GII) RNA, from test samples of food and foodstuffs or food surfaces using real-time RT-PCR. It describes also the processes of virus liberation and concentration from the different matrices, viral RNA extraction (by lysis with guanidine thiocyanate and adsorption on silica) and the specific primers and probe sets. This standard has been technically revised and replaced by ISO 15216: 2017 (https://www.iso.org/standard/65681.html). The method is only applicable to soft fruit, leaf, stem and bulb vegetables, bottled water, bivalve molluscan shellfish, and food surfaces but is not validated for detection of the target viruses in other foodstuffs (including multi-component foodstuffs) or any other matrices, nor for the detection of other viruses in foodstuffs, food surfaces or other matrices.

1.10. Prevention and control of norovirus outbreaks

Despite the increased ability to detect norovirus infections, the challenges to surveillance of norovirus remains as the majority of the people who are infected will not have any contact with medical services and are highly unlikely to have a sample collected for diagnosis (Harris 2016). Norovirus can spread through multiple transmission routes, of which person-to-person and foodborne are the most important (Barclay et al. 2014). The focus of norovirus infection control is to prevent transmission from person-to-person so people are urged not to visit hospitals or their family physicians to prevent the risk of further spread (Harris 2016).

In the absence of an available vaccine and specific antiviral treatment for noroviruses, prevention also involves ensuring that food and water contamination does not occur. Therefore, surveillance systems for foodborne diseases play an important role. Outbreak management and infection control measures are vital to the control of noroviruses (Ong 2013).

Norovirus outbreaks can be prevented or limited by exclusion of infected or shedding food handlers from work until 48–72 hours after recovery, education of food handlers, and standard testing of food handlers during outbreaks (Verhoef et al. 2010).

Careful hand hygiene, through hand washing with water and soap, is probably the single most important method to prevent norovirus infection (Hall et al. 2011). The use of sodium hypochlorite is also very important to disinfect norovirus contaminated surfaces and drinking water (Barclay et al. 2014). In fact, norovirus can be controlled in drinking water by adequate free chlorine disinfection practices with provision of proper pre-treatment processes before chlorination (Katayama and Vinjé 2017). Until recently evaluation of control measures for human norovirus, including disinfection measures has relied primarily on the use of cultivable surrogate noroviruses, such as murine norovirus, feline calicivirus, porcine enteric calicivirus or Tulane virus (Hoezler et al. 2013; Cromeans et al. 2014). But since the successful cultivation of multiple human norovirus strains in stem cell-derived human enteroids (Ettayebi et al. 2016), this model has been used to evaluate the effectiveness of disinfectants on human norovirus infectivity (Costantini et al. 2018). These studies have shown that although 5 minutes of exposure to 70% ethanol and isopropanol slightly reduced viral RNA levels, overall, the alcohols did not inactivate GII.4 viruses (Costantini et al. 2018). These results are in agreement with previous ones that used cultivable surrogate viruses, that based on lack of reduction of viral RNA titers, suggested that GII human noroviruses are not affected by alcohol (Cromeans et al. 2014). However, chlorine treatments showed that complete inactivation of GII.4 strains could be achieved with concentrations as low as 50 ppm (parts per million) (Constantini et al. 2018). These results are consistent with a recent report indicating that treatment with chlorine concentrations <50 ppm were not sufficient to inactivate human norovirus in secondary

effluents from water treatment plants (Kingsley et al. 2017).

The difficulty in identifying norovirus as the causative agent, early in a gastroenteritis outbreak, is one of the major challenges in controlling norovirus outbreaks (Ong, 2013). In fact, testing food contaminated by food handlers that have caused localized point source outbreaks may be inappropriate due to the complexity of the procedure. Testing food is only warranted where implicated food is widely distributed and may be the cause of geographically dispersed outbreaks (Communicable Disease Network Australia, 2010).

Alcohol-based sanitizers used for the control of the transmission of pathogens in general, are relatively ineffective against the human norovirus, reinforcing the need to develop and evaluate new products against this important group of viruses (Liu et al. 2010). It is still controversial the best method for performing effective hand hygiene against norovirus. One key issue is the use of alcohol-based hand rub (ABHR) versus soap and water (Liu et al. 2010). Laboratory studies appear to indicate that ABHR is less active against non-enveloped viruses than soap and the physical rinse with water (Liu et al. 2010). But other epidemiologic studies seem to conclude otherwise (Longtin et al. 2012). Although World Health Organization (WHO) experts recommend the use of ABHR during norovirus gastroenteritis outbreaks (WHO 2009) CDC, states that hand washing with soap and running water reduce norovirus contamination whereas hand sanitizers might serve has an effective adjunct but should not be consider a substitute (Hall et al. 2011).

Permanent standard infection control precautions can minimize the risk of norovirus outbreaks caused by person-to-person transmission or by an infected food handler (Communicable Disease Network Australia 2010). The work practices required to achieve a basic level of infection control include hand hygiene, cough etiquette and routine environmental cleaning. In order to reduce food contamination with norovirus and other foodborne pathogens, it is essential to maintain attention to hand hygiene, prevent cross contamination during food preparation, provision of adequate handwashing facilities for food handlers and ensuring that food handlers do not work while they have symptoms of gastroenteritis. Regardless of the type of outbreak setting three important control measures should be applied in the management of all outbreaks: cleaning and disinfection of contaminated premises, regular handwashing and exclusion and cohorting of ill people (Communicable Disease Network Australia 2010). But the environmental stability of norovirus makes difficult to control norovirus transmission through the usual sanitary measures and the occurrence of a norovirus gastroenteritis outbreaks requires rapid implementation of measures to limit its spread (Friesema et al. 2009; Delacour et al. 2010). Control measures are most effective if implemented within three days of the identification of the initial case (Friesema et al. 2009). Furthermore, public health management of food borne outbreaks will involve identifying and removing the potential food vehicle or source. Providing public health advice is also important to minimize secondary spread

(Communicable Disease Network Australia 2010).

Important approaches to prevent and contain norovirus outbreaks, that are common in several guidelines, and include policies concerning hand hygiene, patient isolation and cohorting (grouping patients based on symptoms), staff exclusion for work, visitor restrictions, enhanced environmental cleaning and disinfection and ward closures (Barclay et al. 2014). Detailed key measures for controlling norovirus outbreaks are described in the table 3.

Table 3. Control guidelines for the prevention and management of norovirus outbreaks

Hand hygiene	Systematic washing by rubbing all hand surfaces vigorously with a mild liquid hand washer for 10-15 seconds under running water. Alcohol-based solutions 79-80% can be used additionally after handwashing with soap and water but should never replace it.
Personal protective equipment	In outbreak settings, should be used if possible whenever cleaning sanitary facilities, vomit or stool and should include gloves, masks, gowns and eyewear.
Cleaning and disinfection	**Environment** Sodium hypochlorite (bleach) at 1000 ppm inactivates norovirus. Frequent touched environmental surfaces such as door handles, bathroom taps, lift buttons, phones and tables should be frequently cleaned.
	Vomit or stools The area should be disinfected with bleach solution. Carpets soiled with stool and vomitus should be washed with warm water, detergent and steam cleaned. They should not be vacuum cleaned to avoid recirculating the norovirus.
	Laundry Contaminated linen, blankets or clothing should be washed as usual in detergent for the maximum washing cycle.
Food	Ill people should not take part in food handling duties and should not return to their usual food handling duties until 48 hours after their symptoms have ceased. Dispose of any exposed food, that is, food that has been handled by an infected person or food that may have been exposed to someone vomiting in close proximity.
Exclusion	Ill people should return to their working places 48 hours after all symptoms have stopped.
Isolation and cohorting	An attempt should be made to separate ill people from non-affected people ('cohorting'), especially if the outbreak setting is in a semi- closed environment and people are required to live in a household-like situation sharing the same facilities.
Visitor restriction	Visiting affected areas should be restricted during the period of an outbreak.
Closure	In some outbreaks that are difficult to control and where there is significant ongoing risk of infection by periodic renewal of the susceptible population, such as cruise ships and camps, it may be necessary to close the facility until it can be cleaned and disinfected properly.

Adapted from Communicable Disease Network Australia, 2011

2. Foodborne Disease Surveillance Systems

WHO states that surveillance systems of foodborne diseases should be given a high priority in the development of food safety infrastructure (WHO 2002). Empowering public health laboratories to conduct laboratory and epidemiologic based surveillance are important global public WHO health objectives. The surveillance systems allows the estimation of foodborne disease burden, the assessment of its relative impact on health and economics, the evaluation of disease prevention and control programs, the rapid outbreak detection and response and are a major source of information for conducting risk assessment, risk management and communication (WHO 2002).

Foodborne disease surveillance includes identifying and controlling outbreaks, gathering data on incidence of these diseases and prevalence of their etiologic agents, vehicles and reservoirs, finding factors at the origin of the outbreak, providing important data for Hazzard Analysis and Critical Control Points (HACCP) systems and risk assessments, estimating impacts of foodborne diseases in health and economics, providing valuable information upon which to base rational food safety program priorities and goals and is necessary for preventing further spread of foodborne diseases (Guzewich et al. 1997). Surveillance of foodborne disease is a continuous and systematic process that consists of receiving notification of illnesses, investigating incidents and reporting findings, collecting and interpreting data, and disseminating information for the effective control of current problems and to provide guidance for preventing the diseases in the future (Guzewich et al. 1997).

In the USA, the National Outbreak Reporting System (NORS) is a web base platform of the CDC (https://www.cdc.gov/nors/) for reporting waterborne, foodborne, and enteric disease outbreaks of all etiologies, including norovirus. This is complemented with another web platform, the CaliciNet, (https://www.cdc.gov/norovirus/reporting/calicinet/index.html) a nationwide electronic surveillance system of local and state public health and regulatory agency laboratories that collects genetic sequences of norovirus strains associated with gastroenteritis outbreaks. The epidemiologic and laboratorial data collected by these two systems were combined by the Norovirus Sentinel Testing and Tracking (NoroSTAT) (https://www.cdc.gov/norovirus/reporting/norostat/index.html) a collaborative network of the CDC in 2012. The more rapid, complete, and integrated reporting by NoroSTAT was considered a key advancement in norovirus outbreak surveillance, providing near real-time monitoring of norovirus outbreak activity and emerging new strains (Shah et al. 2017). In Europe the European Centre for Disease control and Prevention (ECDC) has a toolkit for investigation and response to food and waterborne disease outbreaks with an EU dimension. This toolkit contains documents, guidelines and explanatory texts for international outbreaks investigation and a functionality developed in the software Epidata

(https://ecedc.europa.eu>publications-data>toolkit-investigation). The Netherlands through the National Institute for Public Health and the Environment (RIVM) hosts Noronet (https://www.rivm.nl>noronet), an informal network of scientists working in public health institutes or universities sharing virologic, epidemiological and molecular data on norovirus. The aim of Noronet is to enlarge the knowledge on geographical and temporal trends in the emergence and spread of norovirus variants. In order to ensure standardized typing of sequences, all submitted sequences are typed used the publicly accessible norovirus typing tool.

2.1. Principles underlying the surveillance and management of foodborne disease outbreaks proposed by WHO

The primary goal of surveillance of foodborne disease outbreaks should be the prompt identification of any unusual clusters of disease potentially transmitted through food. Laboratory reporting and disease notification are the main sources used in outbreak detection (WHO 2008).

The investigation of foodborne outbreaks aims to stop both ongoing transmission and similar outbreaks in the future. This can be achieved by the detection and removal of implicated foods, the identification of specific risk factors related to the host, the agent and the environment, the identification of factors that contributed to the contamination, growth, survival and dissemination of the suspected agent, the acquisition of epidemiological data for risk assessment of foodborne pathogens and strengthening of food safety policies (WHO 2008).

A full investigation of a foodborne disease outbreak will normally include epidemiological investigations, environmental and food investigations as well as laboratory investigations (WHO 2008). Epidemiological investigations involve descriptive epidemiology and analytical epidemiological studies. Establishing a case definition, identifying cases and obtaining information from them, analyzing data by time, place and person characteristics, determining who is at risk of becoming ill, developing hypotheses about the exposure/vehicle that caused the disease, comparing the hypotheses with the established facts and deciding whether analytical studies are needed to test the hypotheses are part of the descriptive epidemiology (WHO 2008). On the other hand, analytical epidemiological studies aim to quantify the relationship between specific exposures and the disease under investigation. The two types of analytical studies most commonly used are cohort and case-control studies (WHO 2008).

Environmental and food investigation include identifying the source, mode and extent of the food contamination, assessing the likelihood that pathogens survived processes

designed to kill them or to reduce their numbers, assessing the potential for growth of pathogens during food process storage and identifying and implementing corrective interventions (WHO 2008).

The laboratory investigation requires specific tasks of both clinical and food laboratories. The role of the clinical laboratory should ensure that appropriate clinical specimens are collected, appropriate laboratory investigation of clinical samples are taken and work with other members of the investigation team to identify and characterize the pathogen involved in the outbreak. On the other hand the food laboratory should advise on appropriate samples to be taken from food, perform appropriate laboratory investigations of the food to identify the suspect pathogens, toxins or chemicals, advise on further sampling when a specific agent is found in the food (guiding collection of clinical specimens from food handlers), work with the clinical laboratory to arrange for typing or additional characterization of organisms (serotyping, phage typing, molecular subtyping) and support epidemiological and environmental investigations in detecting the pathogen in the implicated food and understanding how the outbreak occurred (WHO 2008).

In 2017, WHO released a new manual on strengthening surveillance and response to foodborne diseases. This document allows countries to reinforce their current foodborne disease surveillance and response activities and integrate them into existing national surveillance and response systems required by the International Health Regulations (WHO 2017). The manual comprises five modules, an introductory module, two stage-one modules about using indicators and event-based surveillance, to detect foodborne events and investigating foodborne disease outbreaks, one stage-two module, strengthening indicator-based surveillance and a final stage-three module on integrating surveillance data to better understand risks across the food chain (WHO 2017).

3. Norovirus in the Military

Gastrointestinal infections in the USA Armed Forces have consistently been among the most frequent diseases and non–battle injury diagnoses (Hill et al. 2017). Acute gastrointestinal illnesses cause significant morbidity among and degrade the operational effectiveness of USA military members and their units (Chapman et al. 2011; Mccarthy et al. 2000). Despite advances in medicine and improvements in basic sanitation, modern military operations are still affected by gastrointestinal illness. Agents of foodborne illness are an important, preventable cause, of acute gastrointestinal disease. The burden of foodborne

illness caused by specific pathogens among nondeployed active duty USA Army military population has not been determined (Mullaney et al. 2019).

Although gastroenteritis can be bacterial, viral, or parasitic in nature, norovirus has been identified as one of the top five etiologic agents of gastroenteritis among military personnel (Hill et al. 2017). In a recent study, norovirus accounted for 85% of the estimated foodborne illness in nondeployed active duty USA Army personnel, followed by *Campylobacter jejuni*, *Salmonella enterica* (non-typhoidal), *Shigella* spp. and Shiga toxin producing *Escherichia coli* (STEC) non-157. *Campylobacter* and *Salmonella* caused the most bacterial illness (Mullaney et al. 2019). This is in line with studies in England, Wales, Australia and other studies in the USA (Addak et al. 2005, Hall et al. 2005; Scallan et al. 2011; Mullaney et al. 2019).

Among ground troops in the Gulf War, gastroenteritis was the most common illness in soldiers, and 70% of these cases were attributable to norovirus (Hyams et al. 1991). The first reported outbreak of norovirus gastroenteritis in troops occurred during the Operation Desert Shield in Iraq in 1991, (Delacour et al. 2010) although a few years earlier, in 1988, a large nontypical outbreak of Norwalk-like virus affected 48% of 3000 cadets in the USA Air Force Academy (Warner et al. 1991). Norovirus were a major cause of both outbreaks and sporadic disease among crowded USA ground troops in the 1991 war with Iraq (Mccarthy et al. 2000). Noroviruses were also the cause of acute gastroenteritis in other ground and shipboard deployments, and a major cause of morbidity in military forces (Mccarthy et al. 2000).

Norovirus gastroenteritis is a military concern and norovirus outbreaks in military forces are regularly reported. The illness, which is generally mild, may be severe and life-threatening in someone who is already dehydrated due to daily activity (Delacour et al. 2010). The spread of noroviruses in military environment is favored by low infectious dose, prolonged asymptomatic shedding, environmental stability, substantial strain diversity and lack of lasting immunity (Delacour et al. 2010).

Deployed military personnel are at particularly high risk of epidemic gastroenteritis due to crowded conditions that facilitate rapid person-to-person transmission of pathogens. Furthermore, high levels of sanitation are difficult to maintain in hurried combat deployments. Norovirus outbreaks have compromised routine military operations among both shipboard and ground personnel (Mccarthy et al. 2000) and have been associated with significant negative operational impact and degradation of mission readiness (Mayet et al. 2011; Ahmed et al. 2012).

3.1. Epidemiologic data and norovirus disease burden

The full extent of the norovirus gastroenteritis burden in military forces remains unclear. Although noroviruses are frequently mentioned as the cause of gastroenteritis outbreaks in troops deployed overseas, their laboratory diagnosis is rarely done, because diagnostic assays are usually unavailable in deployed microbiology laboratories and clinical specimens are rarely sent to reference laboratories (Delacour et al. 2010).

In 2009, more than 6000 cases of acute gastroenteritis were reported among French troops, most of them being observed in the personnel deployed overseas (Delacour et al. 2010). In a survey of healthy USA service personnel, 77% of those deployed in Iraq and 54% in Afghanistan reported diarrhea at some time during deployment (Sanders et al. 2005).

In the USA surveillance data, spanning from 2005 to 2012, viruses were identified as the most common etiology in acute gastroenteritis outbreaks in military operational settings, with norovirus the most commonly identified (Armed Forces Health Surveillance Center 2013). A recent study among four USA military training facilities identified norovirus as the primary etiology of both sporadic cases and outbreaks of acute gastroenteritis among trainees (Brooks, et al. 2018).

Norovirus outbreaks have been regularly reported in literature in both maritime and land operation theaters as well as in military training establishments in several countries around the world. Tables 4 and 5 summarize the norovirus outbreaks reported among military forces that occurred between 1988-2010 and 2011-2018, respectively.

Table 4. Norovirus gastroenteritis outbreaks in the military 1988-2010

Military Forces	Settings	Date	Number of cases	Outbreak key characteristics	References
USA	Air Force Academy	1988	1440	High attack rate 48%; atypical clinical presentation; *Citrobacter freundii* associated with illness	Warner et al. 1991
USA	Aircraft carrier Mediterranean Sea	1992	585	13% of 4500; lasted 5 weeks; close living conditions	Mccarthy et al. 2000
USA	Aircraft carrier Singapore	1996	784	14% of 5600; Lasted 2 weeks; Close living conditions;	Mccarthy et al. 2000
USA	Aircraft carrier Rhodes, Greece	1996	450	Lasted 13 days;	Mccarthy et al. 2000
USA	Aircraft carrier	1997	1848	Japan, lasted two weeks;	Mccarthy et al. 2000
USA	Training center	1998	99	Food handler point source;	Arness et al. 2000
Israel	Military base	1999	159	Consumption of fresh salad;	Grotto et al. 2004
UK	Deployed troops in Afghanistan	2002	29	Unusual severity; severe outcome in already dehydrated soldiers;	Delacour et al. 2010
UK	Deployed troops in Iraq	2003	1340	Probably origin in locally produced fresh rations; secondary outbreaks in hospital staff	Bailey et al. 2005
Australia	Military base	2007	23	Living conditions may have contributed to the spread of norovirus;	Thomas et al. 2007
USA	Navy ship	2008	65	Attack rate 28,3%; NoV GI and enterotoxigenic *Escherichia coli* in tested samples;	Gonzaga et al. 2011
USA	Military training camp Singapore	2008	156	Suspected environmental contamination; Food handlers tested negative;	Yap et al. 2012
USA	Military base Turkey	2009	187	Multiple NoV and co-pathogens; significant operational impact; civilian community outbreak at the same time;	Ahmed et al. 2012
Germany	Military base	2009	36	Attack rate 12,4%; likely origin in a salad served at the canteen;	Wadl et al. 2010
France	Ship	2010	34	Trimodal curve; Raw mussels and then person-to-person transmission;	De Laval et al. 2011

Table 5. Norovirus gastroenteritis outbreaks in the military 2011-2016

Military Forces	Settings	Date	Number of cases	Outbreak key characteristics	References
France	Military unit	2011	147	Presumed NoV GI based on a single positive sample from a cook; preceded by community outbreak; significant operational impact;	Mayet et al. 2011
USA	Air Force Academy	2011	290	Attack rate 18%; likely introduction by food workers;	Chapman et al. 2011
USA	Army camps (3) Kuwait	2011	345	Enhanced laboratory surveillance enables onsite testing of specimens;	Thompson et al. 2016
Singapore	Military Camps (2)	2013	775	Attack rate: Camp 1-15%; Camp 2-8,3%; Preceded by community; outbreak; NoV G.I.2/GII;	Ho et al. 2015
Peru	Military training center	2013	164	Attack rate: 45.2%; NoV GII;	Ramos et al. 2015
France	Military Unit	2016	103	Attack rate 34%; NoV GII.17; Food worker hand contact;	Sanchez et al. 2017
France	Deployment in Central African Republic	2016	200	Attack rate 22,2%; NoV GIIPe-GII.2, Contaminated food samples with *E. coli;*	Watier-Grillot et al. 2017
Kuwait	USA Military Unit	2018	91	Outbreak lasted 14 days; Laboratory confirmed cases (n=8);	Kebisek et al. 2019

Norovirus outbreaks in military context can sometimes be rather large with high attack rates (outbreak in Peru 2013 or in a military unit in France 2016, with an attack rate of 45.2% and 34%, respectively) and extend for long periods of time, for instance in the outbreak of Kuwait 2018 or the outbreak in an aircraft carrier of the USA in 1992, that lasted for two or five weeks, respectively. It results in a sudden and considerable impact on the daily routines and even in the operational efficiency for example in a USA military base in Turkey in 2008 or in a military base in France in 2011. In some of the outbreaks, noroviruses were initially foodborne or had its origin in contaminated food handlers (the outbreaks in USA 1998, Germany 2009 or France 2016). As close living conditions prevail, secondary transmission further amplifies the number of cases and duration of the outbreaks. Sometimes outbreaks in military settings are preceded by community outbreaks (the outbreak in Singapore, 2013). The knowledge of a norovirus community outbreak should enhance awareness in the military that should reinforce preventive measures in military settings.

3.2. Noroviruses as biologic warfare agents

The Center for Disease Control and Prevention (CDC) and the National Institute of Allergy and Infectious Diseases (NIAID), in conjunction with the US Department of Homeland Security, evaluate the potential threat from various microorganisms and toxins and classified them in three categories, category A, B and C (CDC 2018; NIAID 2018). In category A are the high-priority agents that include organisms that pose a risk to national security because they can be easily disseminated or transmitted from person-to-person, result in high mortality rates and have the potential for major public impact, might cause public panic and social disruption and require special action for public health preparedness. In this category are included smallpox, anthrax, plague, tularemia and viral hemorrhagic fevers. The second highest priority agents are in category B and include those that are moderately ease to disseminate, result in moderate morbidity rates and low mortality rates and require specific enhancements of CDC`s diagnostic capacity and enhanced disease surveillance. Food safety threats, brucellosis, glanders, Q fever, ricin toxin, water safety threats are among the agents/diseases in this group. In the Category C are the third priority agents and include the emerging pathogens that could be engineered for mass dissemination in the future because of availability, ease of production and dissemination and potential for high morbidity and mortality rates and major health impact. Nipah virus and Hantavirus are examples of this agents (CDC 2018).

Several characteristics of noroviruses have led to their classification as category B biodefense agents such as their high infectivity, environmental stability resistance to common disinfectants and ability to cause incapacitating disease (Karst 2010; Koo et al. 2010). Someone who is resistant to one strain may be susceptible to another, given the high genetic diversity of norovirus strains and as infection induces only a short-term homotypic immunity (6–14 weeks), individuals are likely to be repeatedly infected throughout their lifetime (Tan and Jiang 2005; Le Pendu et al. 2006). All of this makes norovirus to be considered the almost perfect pathogen (Hall 2012).

The recent announced method of laboratory culturing of norovirus will surely permit an advance in the research of good decontamination methods and enhance the development of a potential effective vaccine (Bartnicki et al. 2017; Costantini et al. 2018), that in the future could be used by the Military. On the other hand, the possibility of growing norovirus in laboratory may give bioterrorist another potential agent for malicious intents. The fact that norovirus can also be transmitted through air, as hypothetically infectious particles could be aerosolized from vomit or toilet flushing, deposited on the upper deep throat during inhalation and swallowed (Alsved et al. 2019), that there is no available vaccine and that control measures can be difficult to establish in crowded conditions may concur to its utilization as a possible biological weapon.

3.3. Portuguese Army "Gastroenteritis Outbreak Surveillance System"

3.3.1. Organization

Gastrointestinal outbreaks have been registered in the Portuguese Army throughout the XX and the beginning of the XXI centuries. The establishment of the *Laboratório de Bromatologia e de Defesa Biológica* in 2006 allowed laboratory analyses of suspected food at the origin of the outbreaks. While investigating foodborne outbreaks, it was noticed that very often no bacteriologic agent could be identified (Lopes-João 2013), leading to the implementation of molecular diagnostic methods for the identification of norovirus, a suspected cause of foodborne disease outbreaks.

At the same time, Technical Authority Norms were created by the Portuguese Army Veterinary Services in order to enforce the daily sampling of the food produced in the different Army kitchens to normalize the epidemiologic investigation of the outbreaks through the creation of individual and collective questionnaires and to collect biologic samples from the cases in the Army health centers. With the compliance of the Army health services a gastrointestinal infection outbreak surveillance system was established circa 2013.

Army Health Direction (AHD) is the highest health authority in the Portuguese Army, with the responsibility of managing all health services in the Army. AHD supervises the coordination of material resources, personnel and infrastructure in accordance with the directives of the Army Command. AHD has the technical, hierarchical and functional authority on the Army Health Centers (AHC), the Military Veterinary Unit (MVU) and the Biologic and Chemical Defense Laboratory Unit (BCDLU). These Units have a role in the Gastroenteritis Outbreak Surveillance System (GOSS), the MVU with the actions taken by the Epidemiological Surveillance Team (EST) and the BCDLU through the Bromatology and Biologic Defense Laboratory (BBDL) (Figure 5).

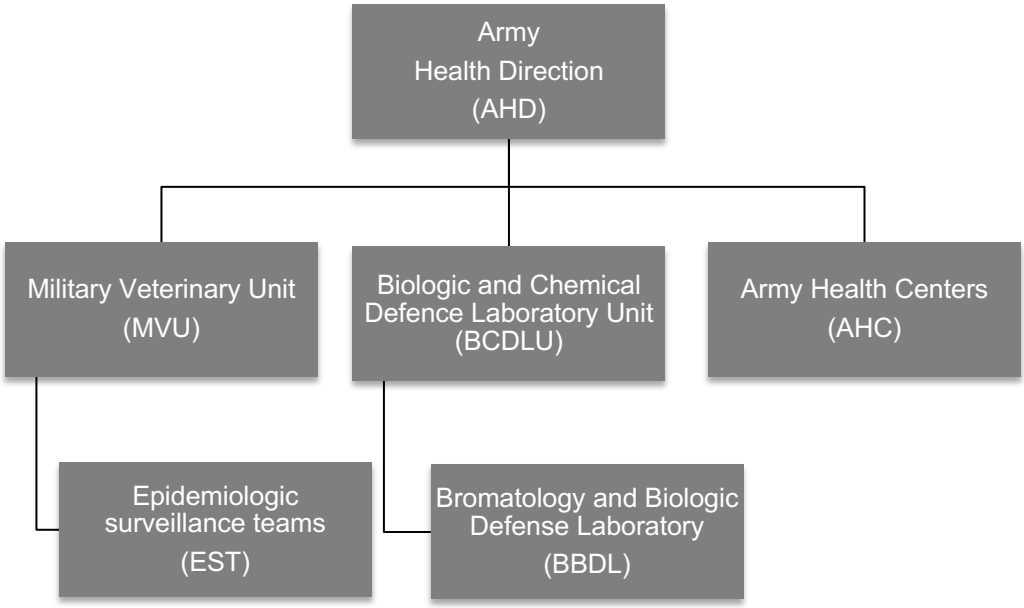

Figure 6. Organigram of the Portuguese Army Health System units and subunits involved in foodborne disease investigations.

Army Health Direction = *Direção de Saúde do Exército*, Veterinary Unit = *Unidade Militar de Medicina Veterinária,* Biologic and Chemical Defense Laboratory Unit = *Unidade Militar de Defesa Biológica e Química*, Army Health Centers = *Centros de Saúde do Exército*, Epidemiologic Vigilance Teams = *Equipas de vigilância Epidemiológica*.

3.3.2. Clinical Investigation

The AHC in the military bases usually detect the outbreak and report to the AHD that informs the MVU, and to the BCDLU. The AHC in the bases perform the clinical diagnosis and treatment of the cases. They are also responsible for the collection of biologic samples, usually stools, used in the identification of the agent responsible for the outbreak.

3.3.3. Epidemiological Investigation

The epidemiologic study is performed by EST from the MVU who are responsible for the distribution of the questionnaires to the cases and also to the controls. They are also responsible for the collection of suspected food and water samples as well as for the evaluation of the food circuits and of the hygienic conditions of the food sector, cuisines, canteens, and food handlers.

3.3.4. Laboratory Investigation

The Laboratory investigation is performed by the BBDL of the BCDLU. Until 2016 BCDLU was also responsible for the epidemiologic investigation, but this activity pass to the MVU after its foundation.

All suspected food items, raw products or prepared meals, water and also swabs collected from the infrastructures, equipment and objects from the food sector or food workers hands are evaluated for hygienic and safety bacteriologic parameters.

Microbiological investigation of stool samples is also performed by the BBDL. The objective is not to do the diagnostic of the clinical cases but use the results for epidemiological purposes as they are fundamental to establish the link between the food or the food handler and the clinical cases of gastrointestinal illness.

The actions taken during a gastrointestinal outbreak in the Portuguese Army are summarized in Figure 6.

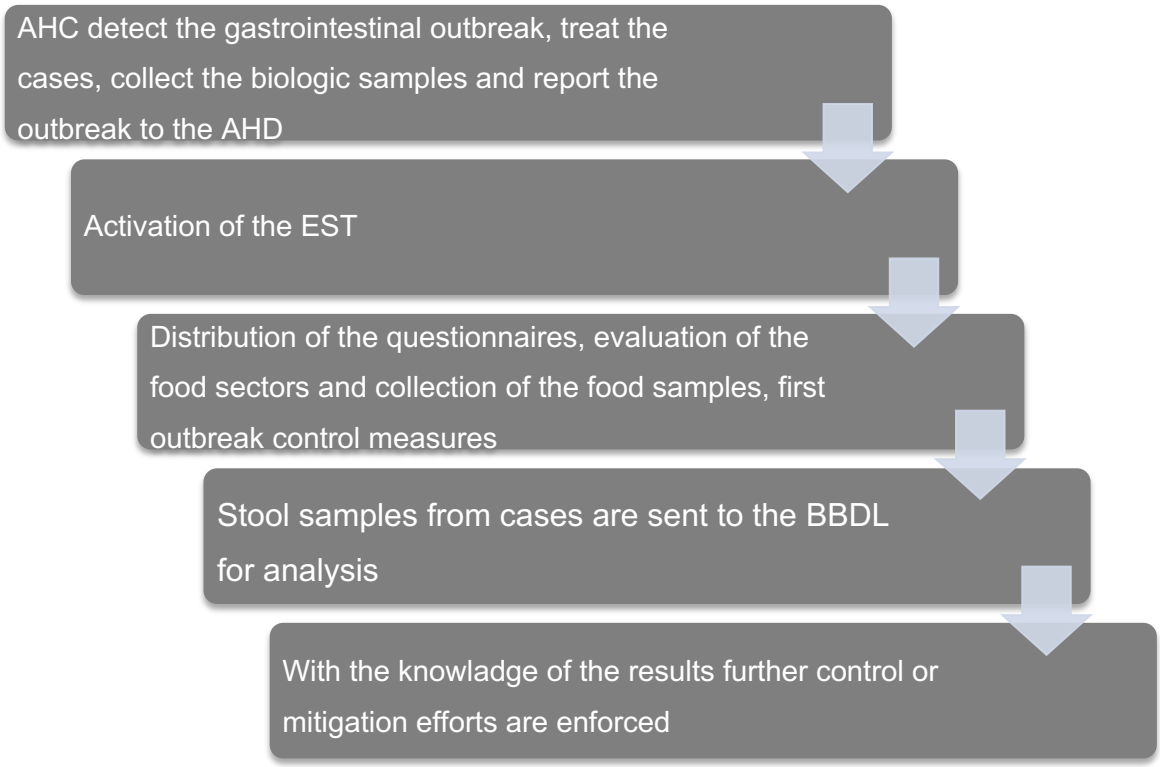

Figure 7. Actions taken in case of a gastrointestinal disease outbreak in the Portuguese Army.

As soon as the results from the different investigations start to emerge, the information is readily reported to the health services and food sectors of the Army settings in order to direct or reinforce the general control measures taken when an outbreak is detected. This information

is directly transmitted to the command of the Units that make sure that actions are taken in order to implement the control measures.

The immediate control measures are usually related to the reinforcement of hygienic conditions in the food sector, the recall of some suspected food and a thoroughly cleaning and disinfection of the sanitary facilities. Additional procedures may include the restrictions to food handlers, isolation of affected individuals in the health services, education in food safety for the workers in the food sector or more profound disinfection actions with chlorinated agents in cuisines, canteens or food warehouses.

II. OBJECTIVES

The main objective of this thesis was to investigate the burden of norovirus gastroenteritis among military in the Portuguese Army. Norovirus are frequently mentioned as a major cause of gastroenteritis outbreaks, particularly in the military population. Nevertheless, little is known about the epidemiological relevance of norovirus in Portugal and specifically in the Portuguese military. Retrospectively, in about half of foodborne outbreaks of acute gastroenteritis registered in the Portuguese Army there is no implication of enteropathogenic bacteria or toxin. We hypothesize that viral agents and more specifically noroviruses are causative of unexplained gastroenteritis outbreaks in the Portuguese military.

This work was developed as a five-year surveillance study that took place between 2013 and 2017 with the following specific objectives:

1. To evaluate the implication of norovirus in acute gastroenteritis cases of the military personnel of the Portuguese Army.

2. To evaluate the role of norovirus in foodborne and waterborne illness.

3. To evaluate the impact of norovirus in sporadic gastrointestinal illness.

4. To ascertain clinical and epidemiological features of norovirus outbreaks occurred in the Portuguese Army.

5. To identify and perform the molecular characterization of the strains of norovirus causing gastroenteritis outbreaks.

III. RESULTS

1. Multiple enteropathogenic viruses in a gastroenteritis outbreak in a military exercise of the Portuguese Army

The present study investigates the cause and the source of an acute gastroenteritis outbreak that occurred during a military exercise of the Portuguese Army, in February 2013. From the total of 160 soldiers that participated in the military exercise 20 developed acute gastroenteritis. A retrospective investigation of the outbreak was performed and stool samples, food items and water were screened for common bacterial and viral agents of foodborne disease. No pathogenic bacteria were found in the stools of soldiers with gastroenteritis however the virological diagnosis revealed the presence of multiple enteropathogenic viruses namely norovirus GI (GI.3), norovirus GII (GII.4 New Orleans 2009), astrovirus and sapovirus as single or co-infection. This was the first report of a viral gastroenteritis outbreak among military personnel in the Portuguese Army.

The content of this section was published in the following paper:

Lopes-João A, Costa I, Mesquita JR, Oleastro M, Penha-Gonçalves C, Nascimento MSJ. (2015) Multiple enteropathogenic viruses in a gastroenteritis outbreak in a military exercise of the Portuguese Army. J Clin Virol. **68**:73-5.

Journal of Clinical Virology 68 (2015) 73–75

Contents lists available at ScienceDirect

Journal of Clinical Virology

journal homepage: www.elsevier.com/locate/jcv

Multiple enteropathogenic viruses in a gastroenteritis outbreak in a military exercise of the Portuguese Army

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ARTICLE INFO

Article history:
Received 11 August 2014
Received in revised form 10 May 2015
Accepted 11 May 2015

Keywords:
Military
Outbreak
Gastroenteritis
Enteropathogenic viruses

ABSTRACT

Background: Gastroenteritis is one of the most common infectious diseases in the military populations and can diminish operational effectiveness and impede force readiness.
Objectives: The present study investigates the cause and the source of an acute gastroenteritis outbreak that occurred during a military exercise of the Portuguese Army, in February 2013.
Study Design: A retrospective investigation was performed and stool samples, food items and water were screened for common foodborne bacteria and viruses, namely *Norovirus* GI, *Norovirus* GII, Astrovirus, *Rotavirus*, Adenovirus and *Sapovirus*.
Results: From the total of 160 soldiers that participated in the military exercise 20 developed gastroenteritis (attack rate of 12.5%). Symptoms were predominantly vomiting ($n = 17$, 85%) and diarrhoea ($n = 9$, 45%). The first cases occurred 24–48 h after drinking water from the creek, the plausible origin of the outbreak. The epidemic peak was registered 2 days after and the last cases 6 days after, upon returning to base. No pathogenic bacteria were found in stools however virological analysis revealed the presence of multiple enteropathogenic viruses, namely Norovirus GI (GI.3), Norovirus GII (GII.4 New Orleans 2009), Astrovirus and Sapovirus, as single or co-infections. Food and water samples were not tested for the presence of viruses due to exhaustion of samples on bacteriological analysis.
Conclusions: To the best of our knowledge this is the first report of a viral gastroenteritis outbreak among military personnel in the Portuguese Army.

1. Background

Gastroenteritis is one of the most common infectious diseases in the military populations [1]. It can be caused by bacterial, viral or parasitic pathogens and the majority of cases are associated to the consumption of contaminated food or water [1]. Despite the implementation of health protection measures (e.g., provision of clean water, safe food) gastroenteritis continues to be an important cause of morbidity among military forces [1]. In 2012 diarrhoeal diseases were responsible for over 17,000 healthcare encounters affecting over 15,000 service members in active U.S. Armed Forces [2]. Military environments, where people live in close proximity and share the same facilities, are particularly at-risk for acute gastroenteritis outbreaks, namely those caused by *Norovirus*. As such, *Norovirus* outbreaks have been reported in both maritime and land theaters of operations as well as in military training establishments in Forces from the USA, Israel, United Kingdom, Australia, Germany and France [3–9]. Moreover, *Norovirus* was found to be the most common cause of gastroenteritis in the USA marines during Operation Iraqi Freedom [8] and a common cause of outbreaks among deployed British troops [9]. Although generally considered mild and of short duration in healthy young adults (e.g. soldiers),

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http://dx.doi.org/10.1016/j.jcv.2015.05.008
1386-6532/

Norovirus gastroenteritis can diminish operational effectiveness and impede force readiness, as demonstrated by an array of military units in Iraq [8,10,11].

2. Objectives

The present study investigates the cause of an acute gastroenteritis outbreak in military personnel of the Portuguese Army during a military exercise in Portugal in February 2013

3. Study design

3.1. Epidemiological investigation

On the 18 of February 2013, the Portuguese Army Laboratory of Bromatology and Biological Defence was notified by the medical service attending the military exercise involving 160 troops of an acute gastroenteritis outbreak that started on February 14. The Exercise started on February 11 and lasted until February 18. Based on the symptoms the possibility of a foodborne outbreak was considered and an investigation was initiated. Stool samples, food items and water were immediately collected and a retrospective investigation was initiated to identify the cause and source of infection, as well as the mode of transmission. A questionnaire was conducted to gather epidemiological and clinical data. The questionnaire was anonymous. Respondents were asked to list clinical symptoms and disease onset. A case was defined as a member of the military unit staff who presented, in the period between February 14 and 20, diarrhoea (three or more liquid stool in 24 h) or a vomiting episode plus fever (temperature ≥38 °C).

3.1.1. Analysis of food items and water

Field rations served to the military personnel were assessed for food safety and hygiene through bacteriological studies. Standard methods were employed to identify possible food contamination. Food items were tested for *Salmonella* spp. (Vidas® *Salmonella* (SLM), bioMerieux), Enterobecteriacea (ISO 21528-2:2004), *Escherichia coli* (ISO 16649-2:2001), *Bacillus cereus* (ISO 7932:1987), *Clostridium perfringens* (ISO 7937:1997), *Campylobacter* spp. (Vidas® *Campylobacter* (CAM), bioMerieux), *Staphylococcus aureus* (ISO 6888-1:1999) and *Listeria monocytogenes* (Vidas® *Listeria monocytogenes* II, bioMerieux). Water sample drawn from one camel back, filled in a mountain creek, was processed according to standard membrane filter procedures for enumeration of culturable microorganisms (ISO 6222:1999), *E. coli* (ISO 9308-1:2000), coliform bacteria (ISO 9308-1:2000), *Pseudomonas aeruginosa* (ISO 16266:2006), faecal *Streptococci* (ISO 7899/2:1984) and detection and enumeration of the spores of sulphite-reducing anaerobes (Clostridia) (ISO 6461-2:1986). Neither food nor water were searched for the presence of viruses due to the exhaustion of samples on bacteriological analysis.

3.1.2. Analysis of stool samples

Only seven stool samples from soldiers with gastroenteritis were available for analysis (one sample per soldier). Stools were screened for common foodborne bacteria, namely *Shigella*, *Salmonella*, *Campylobacter*, *Vibrio*, *B. cereus* using standard coproculture methods [12].

Stools were also searched for enteropathogenic viruses, namely *Norovirus* GI, *Norovirus* GII, Astrovirus, *Rotavirus*, Adenovirus and *Sapovirus*. For viral detection, stools were diluted (10%) in phosphate-buffered saline, pH 7.2, solids were removed by centrifugation at 13500 × *g* for 1 min and viral nucleic acid was extracted from 140 μl of each clarified stool suspension using the Magmax kit (Ambion, TX, USA) according to the manufacturer's instructions. A multiplex real-time PCR for detection of *Norovirus* GI, *Norovirus* GII, Astrovirus, *Rotavirus*, Adenovirus *and Sapovirus* including an internal control (FTD Viral Gastroenteritis, Fast Track Diagnostics, Luxembourg) was used according to the manufacturer's instructions.

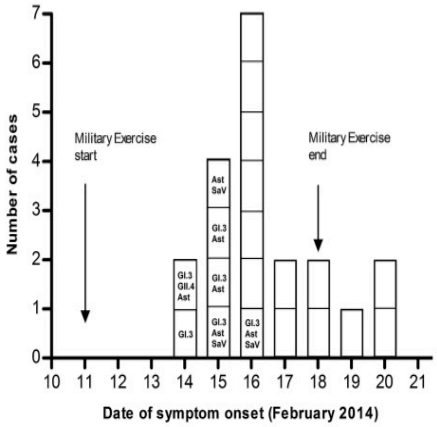

Fig. 1. Cases associated with an outbreak of gastroenteritis (*n* = 20) in the military exercise of the Portuguese Army, February 2013, by date of onset of symptoms. GI.3 (*Norovirus* GI.3); GII.4 (*Norovirus* GII.4); Ast (Astrovirus); SaV (*Sapovirus*).

Stool samples positive for *Norovirus* were subsequently sequenced and typed. Briefly, after two conventional RT–PCR targeting the capsid gene (171 nt for GI, 253 nt for GII) [13] and a contiguous genomic region sequence comprising both the polymerase and the capsid regions of *Norovirus* GII (1.325 bp) [14] amplified products of the expected sizes were sequenced (Sequencer Analyser ABI-Prism 3130 xl, PE Applied Biosystems). Genotypes were assigned using an automated genotyping tool implemented at the National Institute for Public Health and the Environment (RIVM) [15].

4. Results

From the total of 160 soldiers that participated in the military exercise, 20 met the case definition for gastroenteritis, yielding an overall attack rate of 12.5%. The symptoms were predominantly vomiting (*n* = 17, 85%) and diarrhoea (*n* = 9, 45%). From the 20 militaries, 6 (30%) reported having both diarrhoea and vomit.

The soldiers had eaten field rations (lyophilized or sterile) and drank potable water, however some have reported drinking water from a water creek. The first cases (*n* = 2, 10%) occurred on 14 February, 24–48 h after drinking water from the creek, and three days after the beginning of the military exercise (Fig. 1).

The epidemic peak was registered in 16 February (*n* = 7, 35%) and the last cases (*n* = 2, 10%) were observed on 20 February, two days after returning to base.

Field rations showed no pathogenic bacteria or fecal indicator (*E. coli*), and total viable count was <10CFU/g. On the contrary the camel back drinking water showed a high total viable count of 5.8×10^3 CFU/ml, although no fecal indicators (*E. coli*, coliform bacteria, *P. aeruginosa*, faecal *Streptococci* and spores of sulphite-reducing anaerobes) were found. Food or water were not searched for the presence of enteric viruses due to the exhaustion of samples on bacteriological analysis.

Among the 7 stool samples that were available for analysis, all tested negative for pathogenic bacteria. However all samples showed to be positive for at least one enteropathogenic virus, namely *Norovirus* GI, GII, Astrovirus or *Sapovirus* (Fig. 1). No *Rotavirus* or Adenovirus were found. *Norovirus* GI was detected in

6 of the 7 stools. In 1 of those 6 *Norovirus* GI positive samples, GI was detected alone while in the remaining 5 multiple viruses were co-detected (2 samples *Norovirus* GI + Astrovirus + *Sapovirus*; 1 sample *Norovirus* GI + *Norovirus* GII + Astrovirus; 2 samples *Norovirus* GI + Astrovirus). The only sample with no *Norovirus* GI showed to be positive for both Astrovirus and *Sapovirus*. Molecular characterization of the GI/GII *Norovirus* isolates identified 5 as being GI.3 and 1 as being GII.4 New Orleans 2009. All the GI.3 isolates showed 100% similarity.

5. Discussion

The present work describes the investigation of an outbreak of gastroenteritis that occurred in February 2013, during a military exercise in Portugal that affected 12.5% of the soldiers.

The involvement of pathogenic bacteria in this outbreak was ruled out since stools tested negative for *Shigella*, *Salmonella*, *Campylobacter*, *Vibrio* and *B. cereus*. However, virological analysis revealed the presence of multiple viruses (*Norovirus* GI, *Norovirus* GII, Astrovirus and *Sapovirus*), as single or co-infections.

Given the severity of the gastroenteritis presented by the soldiers (healthy young adults) it is tempting to speculate that the primary cause of disease were *Noroviruses*. Astrovirus and *Sapovirus* can alone cause acute gastroenteritis, however these viruses are primarily linked to severe acute disease in infants, young children, elderly and immuno compromised [16]. The co-infection Astrovirus/*Sapovirus* in the only soldier with no *Norovirus* excretion could have resulted in increased pathology and severity of illness, explaining the acute gastroenteritis.

The source of this outbreak remains uncertain, since food and water samples were not searched for the presence of viruses. However, unlike food items, the creek water drawn from the camel back had a low bacteriological quality, as indicated by the high total viable count, suggesting a potential implication in the outbreak. Moreover, soldiers presenting gastroenteritis reported having drunk water from the camel back filled with water from the creek, 24–48 h before the onset of disease, corresponding to the typically short incubation period of the detected enteropathogenic viruses.

Having detected GI.3 strengthens the hypothesis of a waterborne origin. In fact, *Norovirus* GI strains have been reported as the most often implicated *Norovirus* genogroup in waterborne outbreaks [17].

Person-to-person transmission may have also played a role in this outbreak since the last cases appeared 6 days after the beginning of the outbreak suggesting that the later cases were not exposed to the same source as the first ones. Moreover, the lack of hygienic conditions in the military exercise might have favoured the transmission. Viral gastroenteritis illness is usually mild and self-limited in healthy adults. However, in troops experiencing physically demanding daily activities disease may be severe due to added dehydration, as in this outbreak where the two more severe cases required hospitalization and intravenous fluid therapy.

The present work highlights that the consumption of water of uncertain origin is a risk behavior and should be avoided at all means, specially in troops where gastroenteritis can substantially diminish operational effectiveness.

To the best of our knowledge this is the first report of a viral gastroenteritis outbreak among military personnel in the Portuguese Army.

Funding

Not applicable.

Competing interests

The authors have no competing interests.

Ethical approval

Not applicable.

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2. Acute gastroenteritis outbreak associated to norovirus GI.9 in a Portuguese Army base

This work describes the investigation of an outbreak of acute gastroenteritis that occurred in a base of the Portuguese Army, in April 2015. From a total of 938 military personnel stationed in the base 46 developed acute gastroenteritis. The study is focused on the epidemiological curve, symptoms experienced by the affected soldiers and the results of food, water and stool microbiological analysis. Stool analysis of seven soldiers with gastroenteritis showed to be positive for norovirus GI.9 suggesting it was the probable cause of the outbreak. This report shows that genogroup I norovirus can also cause considerable morbidity in healthy young soldiers, affecting the operational effectiveness of military forces.

The content of this section was published in the following paper:

Lopes-João A, Mesquita JR, de Sousa R, Oleastro M, Penha-Gonçalves C, Nascimento MS. (2017) Acute gastroenteritis outbreak associated to norovirus GI.9 in a Portuguese Army base. J Med Virol. **89** (5):922-925.

Journal of Medical Virology 89:922–925 (2017)

Acute Gastroenteritis Outbreak Associated to Norovirus GI.9 in a Portuguese Army Base

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Gastroenteritis is considered a major illness within the military settings being caused by foodborne enteric pathogens that are particularly easily spread in the crowded conditions of military camps. Gastroenteritis outbreaks caused by norovirus usually affect a great number of soldiers due to the low infectious dose, copious viral shedding, and environmental stability. The present study describes the investigation of an outbreak of acute gastroenteritis that occurred in April 2015 in a Portuguese army base, focusing on the study of the epidemiological curve, symptoms experienced by the affected soldiers, and results of food, water, and stool microbiological analysis. From a total of 938 military personnel stationed on the base 46 soldiers developed acute gastroenteritis. Stool analysis of seven cases showed to be positive for norovirus GI.9 that was the probable cause of the outbreak. This report shows that genogroup I norovirus can also cause considerable morbidity in healthy young soldiers, affecting the operational effectiveness on the military forces. ***J. Med. Virol. 89:922–925, 2017***.

KEY WORDS: military; outbreak; gastroenteritis; norovirus

INTRODUCTION

Gastroenteritis is considered a major illness within the military settings being caused by foodborne enteric pathogens that are particularly easily spread in the crowded conditions of military camps [AFHSC, 2013a]. Many efforts have been employed to reduce food-related illness with special focus on the employment of protective measures, education of good food handling practices, and the implementation of early-warning systems. However, gastroenteritis remains a central concern in morbidity among military forces and of all the enteric pathogens, noroviruses were found to be the most common cause of gastroenteritis in both U.S. and U.K. troops [Bailey et al., 2008; AFHSC, 2013a,b]. Gastroenteritis outbreaks caused by norovirus usually affect a great number of people due to the low infectious dose, copious viral shedding, and environmental stability [Hall, 2012]. The high morbidity rendering a high number of military personnel non-effective, diminishing operational effectiveness, and impacting on force readiness [Thornton et al., 2005; Bailey et al., 2008]. The present study describes the investigation of an outbreak of acute gastroenteritis that occurred in April 2015 in a Portuguese army base, focusing on the study of the epidemiological curve, symptoms experienced by the affected soldiers, and results of food, water, and stool microbiological analysis.

MATERIALS AND METHODS

Epidemiological Investigation

On April 23, 2015, the Portuguese Army Laboratory of Bromatology and Biological Defence (LBBD) was

Conflict of interest: The authors have no funding or conflicts of interest to disclose.

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Accepted 3 October 2016

DOI 10.1002/jmv.24704
Published online 26 December 2016 in Wiley Online Library (wileyonlinelibrary.com).

notified of an acute gastroenteritis outbreak in a military base that started 3 days before (20 April). Stool specimens, food items, and water were collected during the outbreak and a retrospective investigation was initiated. An anonymous questionnaire was conducted to gather epidemiological and clinical data asking soldiers to list clinical symptoms and disease onset. A case was defined as a member of the military unit staff who presented, in the period between April 20 and 24, 2015, at least one measurable symptom of gastroenteritis (three or more liquid stool in 24 hr, a vomiting episode or temperature $\geq$37°C), have eaten at the base canteens, and have attended the base medical center. The base had a total staff of 983 military personnel divided in several units, each with its own canteen (A, B, C) and cuisine, where food meals were prepared and served. Food items used in the three canteens had the same origin, but were prepared by different cooking teams. Soldiers were not allowed to eat in canteens from other companies.

As per standard procedures in the military bases, samples of served food are routinely stored in the refrigerator during a 48-hr period for future testing, if required. As notification to LBBD occurred 3 days after the first symptomatic case, food samples were no longer available for microbiological analysis. In an effort to investigate the origin of the outbreak and despite having passed 3 days from the index case (April 20), the LBBD collected samples from the dinner served April 21 in canteen A and the meals served the April 23 and 24 of canteen B. No food was sampled from canteen C because no cases were reported there. Tap water samples were collected from canteen A and B on the April 24. Stools from seven symptomatic soldiers (Four eating in canteen A and three in canteen B) were also collected: six individual specimens on April 23 and one on April 24.

Laboratory Investigation

Food samples were tested for bacteria namely, *Salmonella* spp. (Vidas® Salmonella [SLM], bioMerieux, Marcy-l'Etoile, France), *Enterobacteriaceae* (ISO 21528-2:2004), *Escherichia coli* (ISO 16649-2:2001), *Bacillus cereus* (ISO 7932:1987), *Clostridium perfringens* (ISO 7937:1997), *Campylobacter* spp. (Vidas® *Campylobacter* (CAM), bioMerieux), *Staphylococcus aureus* (ISO 6888-1:1999), and *Listeria monocytogenes* (Vidas® *L. monocytogenes* II, bioMerieux). Water was processed according to standard membrane filter procedures for enumeration of culturable microorganisms (ISO 6222:1999), *E. coli* (ISO 9308-1:2000), coliform bacteria (ISO 9308-1:2000), *Pseudomonas aeruginosa* (ISO 16266:2006), faecal Streptococci (ISO 7899/2:1984), and detection/enumeration of spores of sulfite-reducing anaerobes (*Clostridia*) (ISO 6461-2:1986). Neither food nor water was tested for the presence of enteric viruses due to the exhaustion of samples in bacteriological analysis.

Stools were screened for common enteropathogenic bacteria, namely *Shigella*, *Salmonella*, *Campylobacter*, *Vibrio*, *B. cereus* using standard coproculture methods. Counting of *C. perfringens* in stools was also performed. Stools were also searched for enteropathogenic viruses, namely norovirus, astrovirus, rotavirus, adenovirus, and sapovirus using a multiplex Real-Time PCR assay (FTD Viral Gastroenteritis, Fast Track Diagnostics, Luxembourg) as previously described [Lopes-João et al., 2015]. Stool samples that showed to be positive for norovirus were submitted to conventional RT-PCR assays, and subsequently sequenced and typed. Briefly, after two conventional RT-PCR targeting a 747 bp fragment of ORF1 gene that encodes a region of the RNA-dependent RNA polymerase (RdRp) and a 268 bp fragment of the ORF2 gene that encodes a region of the capsid protein [Vinjé et al., 2004; Fonager et al., 2013], amplified products of the expected sizes were sequenced (Sequencer Analyser ABI-Prism 3130 genetic analyzer, Applied Biosystems, LLC, Foster City, CA). Genotypes were assigned using a public automated genotyping tool (http://www.rivm.nl/en/Topics/N/NoroNet/Databases/Sequence_typing_tool) from Noronet platform developed by the National Institute for Public Health and the Environment (RIVM) [Kroneman et al., 2011].

RESULTS AND DISCUSSION

From the total of 938 military personnel that were stationed on the base 46 soldiers met the case definition, 26 of which have eaten at canteen A and 20 at canteen B.

The first case of this outbreak occurred on April 20 in a soldier that ate on canteen B (Fig. 1). On this canteen, the majority of cases occurred on the April 22 (nine cases) being the last cases reported on the April 24 (two cases). Regarding canteen A, the first case occurred in April 21, the majority of cases occurred on April 23 (16 cases), and the last cases were reported on April 24 (four cases). The bacteriological analysis of the food served at canteen B indicated good microbiological quality but dinner served at canteen A (baked pork meat with saffron sauce and rice) presented an enumeration of *C. perfringens* of 1.8×10^6 CFU/g, a level above the acceptable microbiological limits and considered potentially hazardous. However, the batch of frozen raw pork and saffron used to prepare this dinner presented microbiological values within acceptability, with no *C. perfringens* detected, suggesting that the high *C. perfringens* levels founded in baked pork meat was likely caused by inadequate food handling practices.

The bacteriological analysis of the tap water from canteens A and B, that was only sampled at the end of the outbreak (on April 24), was of acceptable quality.

However, we found that an additional water source for the canteens that used groundwater well that was positioned inside the base had been consumed until April 23 and showed an interesting connection to the

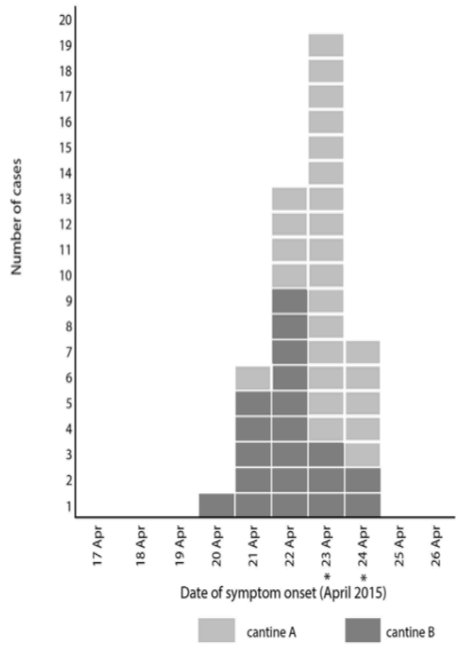

Fig. 1. Norovirus GI.9 gastroenteritis cases (n = 46) by date of onset of symptoms related with two canteens of a military base, Portugal, April 20–24 of 2015. *Dates in which stool specimens were collected.

outbreak. Canteen B, that registered the first case of the outbreak, distanced approximately 1 km from this groundwater well, while canteen A (that registered cases 24 hr later) distanced approximately 2 km, and canteen C (with no cases) approximately 3 km. We could not ascertain whether distance-factor was involved in the transport of a waterborne enteropathogenic agent from the point source (the groundwater well) with the consequent concentration decrease of the contaminated water along the length of the pipes. However, the time difference in the appearance of first case in canteens B versus A, as well as the peaks and last cases, could be justified by the closest proximity of the well to canteen B, compared to canteen A. This hypothesis is supported by the absence of cases in the furthest canteen C (3 km). Unfortunately, samples from the groundwater well were not provided for analysis.

Among the seven stool specimens that were available for analysis only one presented high *C. perfringens* count (2.6×10^7 CFU/g) that could be considered of clinical significance ($>10^6$ CFU/g). This stool was from a soldier that had dinner at canteen A, where the pork meat with high *C. perfringens* counts was served. Since high counts were only found in the stools of one soldier, *C. perfringens* can be ruled out as the cause of the outbreak. Concerning enteric viruses detection, all seven stool specimens showed to be positive for norovirus GI. No other enteric viruses were found in these stools. Subsequent genotype characterization in both partial ORF1 and ORF2

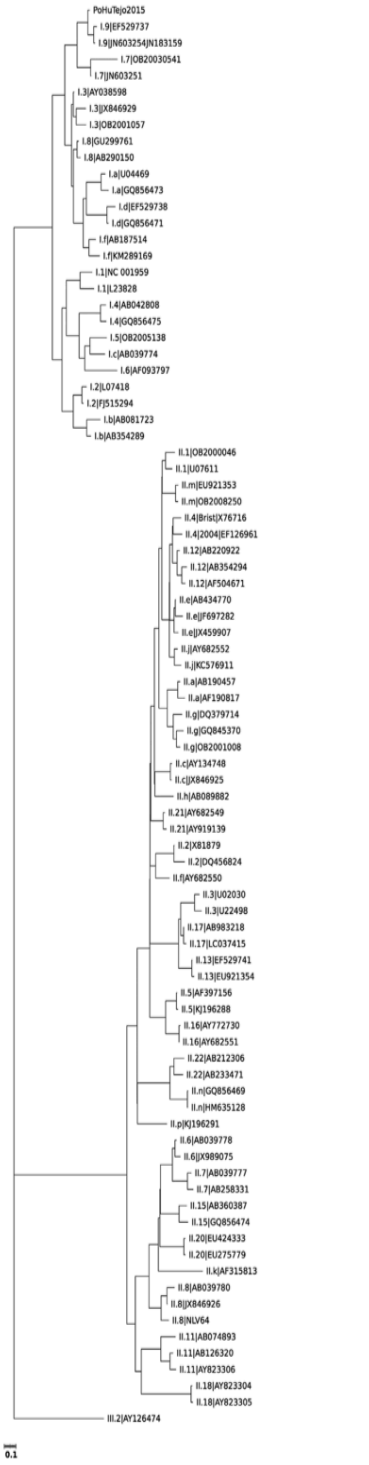

Fig. 2. Phylogenetic analyses of PoHuTejo2015 (PoHu, Portuguese Human Specimen/geographic region/date). Partial region of polymerase gene (ORF1, 747 bp; 4,627–5,357). Tree was constructed using the typing tool from Noronet.

regions identified all isolates as GI.9 (Figs. 2 and 3), with 100% nucleotide sequence homology, suggesting that this GI.9 strain was the most probable cause of the gastroenteritis cases of both canteens and that all cases had the same origin. The contiguous genomic region sequence assembled using the amplified nucleotide sequences of the polymerase and capsid regions has been deposited in GenBank under accession number KX458103.

This investigation was intricate because the outbreak affected soldiers that ate at different canteens, each with its own team of food handlers. Moreover, the exhaustion of samples on bacteriological analysis (traditionally the first pathogens to be searched) did not left any food or water available for virological analysis that could have allowed a definite conclusion on the origin. Poor hygienic manipulation of food was suspected in canteen A based on the presence of *C. perfringens* in the meal and its absence in the raw materials used to prepare it. However, this outbreak cannot be ascribed to poor hygiene in food handling in canteen A, since each canteen has its own staff and the bacteriological analysis of the food served at canteen B indicated good microbiological quality. It would be tempting to attribute the norovirus GI.9 transmission to an infected food handler, but this is highly unlikely since different cooking teams prepared the food (one in each unit/canteen) and soldiers were not allowed to eat in canteens from other companies.

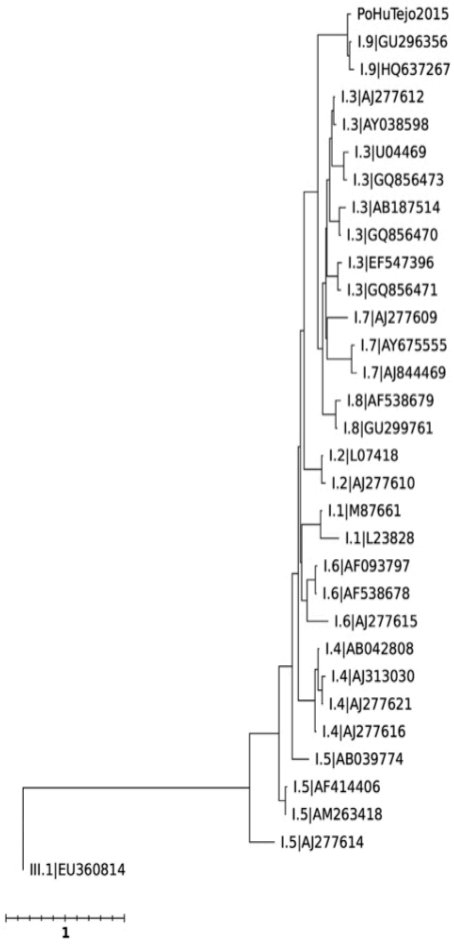

Fig. 3. Phylogenetic analyses of PoHuTejo2015 (PoHu, Portuguese Human Specimen/geographic region/date). Partial region of the capsid gene (ORF2, 268 bp; 5,357–5,622). Tree was constructed using the typing tool from Noronet.

Based on all the data, it is tempting to speculate that this GI.9 norovirus outbreak might have been waterborne and that the groundwater that was supplied to the canteens could be the point source of the norovirus contamination. Unfortunately, this hypothesis cannot be confirmed, since the analyzed water was only sampled at the end of the outbreak, after the alert, and adjustment of disinfection measures. This report shows the importance of continuous epidemiological surveillance and swift intervention in military settings, as well as the impact of norovirus GI.9 in the morbidity of military personnel.

ACKNOWLEDGMENT

None.

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3. Country-wide surveillance of norovirus outbreaks in the Portuguese Army

The present study documents the epidemiological, clinical and laboratory investigations of four norovirus gastroenteritis outbreaks that occurred in several Portuguese Army settings in mainland Portugal and the Azores archipelago, between October 2015 and October 2017. In this short two-year surveillance period a total of 99 soldiers were affected among the 618 stationed in the implicated base units or involved in military exercises. Noteworthy, 30 soldiers had to receive treatment at the military hospital due to severity of symptoms. From the total of 27 stool samples studied, 20 were positive for norovirus. Phylogenetic analysis showed that the noroviruses involved in the four outbreaks were all genogroup II, namely GII.17, GII.Pe-GII.4 Sydney 2012, GII.P2-GII.2 and GII.P16-GII.2. Overall, this study shows that noroviruses have a major role in gastroenteritis outbreaks in the Portuguese Army, both in the stationed and in exercise context.

The content of this section was published in the following paper:

Lopes-João A, Mesquita JR, de Sousa R, Oleastro M, Penha-Gonçalves C, Nascimento MS. (2018) Country-wide surveillance of norovirus outbreaks in the Portuguese Army, 2015-2017. J R Army Med Corps. **164** (6):419-422.

Country-wide surveillance of norovirus outbreaks in the Portuguese Army, 2015–2017

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Received 28 May 2018
Accepted 17 July 2018

To cite: Lopes-João A, Mesquita JR, de Sousa R, *et al*. *J R Army Med Corps* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/jramc-2018-000991

ABSTRACT

Introduction Gastrointestinal infections are among the most common foodborne and waterborne diseases in military populations, with direct implications in operational efficiency and force readiness. Through the surveillance system of reportable acute gastrointestinal illness in the Portuguese Army, four norovirus outbreaks were identified between October 2015 and October 2017 in mainland Portugal and the Azores archipelago. The present study documents the epidemiological, clinical and laboratory investigations of these norovirus outbreaks.

Methods Cases were investigated and epidemiological questionnaires were distributed to all soldiers in each military setting where the outbreaks occurred. Stool samples from soldiers with acute gastroenteritis illness were collected and screened for common enteropathogenic agents. Food and water samples served on the settings were also collected for microbiological investigation. Norovirus-positive samples were further characterised by sequence analysis using a public automated genotyping tool.

Results The four outbreaks affected a total of 99 soldiers among the 618 stationed on base units and in a military exercise. A total of 27 soldiers provided a stool sample, of which 20 were positive for norovirus by real-time PCR. Phylogenetic analysis showed that the noroviruses involved were all genogroup II, namely GII.17, GII.Pe-GII.4 Sydney 2012, GII.P2-GII.2 and GII.P16-GII.2. Of note, 30 soldiers had to receive treatment at the military hospital due to severity of symptoms.

Conclusion In this short, two-year surveillance period, a total of four norovirus gastroenteritis outbreaks were detected in the Portuguese Army which caused a considerable morbidity, showing once again the impact of norovirus on Army effectiveness and force readiness.

Key messages

- Four norovirus outbreaks were identified in the Portuguese Army between October 2015 and October 2017.
- The outbreaks affected a total of 99 soldiers.
- Phylogenetic analysis showed that the noroviruses involved were all genogroup II, namely GII.17, GII.Pe-GII.4 Sydney 2012, GII.P2-GII.2 and GII.P16-GII.2.
- Thirty soldiers had to receive treatment at the military hospital due to severity of symptoms.

INTRODUCTION

Gastroenteritis is considered a major illness within the military settings, being caused by foodborne enteric pathogens, which are particularly easily spread in the crowded conditions of military camps.[1] Great efforts have been done to reduce food-related illness, with special focus on the employment of protective measures, education on good food handling practices and implementation of early warning systems; however, gastroenteritis continues to be an important cause of morbidity among military forces.[1 2] Noroviruses were found to be the most common cause of gastroenteritis outbreaks in the military,[1–3] but bacteria have been reported as a prevalent cause of traveller's diarrhoea among deployed military personnel.[4] In the Portuguese Army noroviruses also seem to play a major role in gastroenteritis outbreaks in soldiers, both stationed or in an exercise context.[5 6] Besides transmission through food and water, person-to-person transmission is very common since the environmental stability of the virus even in minimal amounts is sufficient to affect a great number of soldiers.[1 2] The high morbidity interferes with induction and training schedules, rendering a high number of soldiers non-effective, diminishing operational effectiveness and impeding force readiness.[3 7] The present study documents the epidemiological, clinical and laboratory investigations of four norovirus outbreaks that occurred in Portuguese military settings between October 2015 and October 2017.

MATERIALS AND METHODS

Epidemiological investigation

Between October 2015 and October 2017, gastroenteritis outbreaks that occurred in the base units and in military exercises in mainland Portugal and islands were registered through the surveillance system of reportable acute gastrointestinal illness of the Portuguese Army. Settings were visited and epidemiological and clinical questionnaires were distributed to soldiers. A probable case of acute gastroenteritis illness (AGI) was defined as a person who experienced acute diarrhoea (three or more episodes of loose stools in a 24-hour period) or presented with vomiting with at least one of the following symptoms: one or more episodes of loose stools in a 24-hour period, abdominal cramps, headache, muscle aches or fever. A norovirus AGI confirmed case was a probable case with a stool sample positive for norovirus.

Microbiological investigation of stools

Stools were collected from soldiers who fulfilled the definition of probable AGI case and were sent to the Portuguese Army Laboratory of Bromatology and Biological Defence (LBBD). Stools were analysed for common enteropathogenic bacteria, namely *Shigella*, *Salmonella*, *Campylobacter*, *Vibrio* and *Bacillus cereus*, using standard coproculture methods. Counting of *Clostridium perfringens* spores was also performed. Stools were also screened for a panel of enteric viruses, namely norovirus, astrovirus, rotavirus, adenovirus and sapovirus, using a commercial multiplex real-time PCR (RT-PCR) (FTD Viral Gastroenteritis, Fast Track Diagnostics, Luxembourg) according to the manufacturer's instructions. Stools positive for norovirus were further characterised by conventional RT-PCR and sequencing, as previously described.[8 9] Positive and negative controls were included in all RT-PCR reactions.

Phylogenetic analysis of open reading frame (ORF)1 and ORF2 sequences and genotype assignment was performed using a public automated genotyping tool from the NoroNet platform curated by the National Institute for Public Health and the Environment (RIVM).[10]

Microbiological investigation of food and water

Food samples were tested for *Salmonella* spp (Vidas Salmonella (SLM), bioMérieux), Enterobacteriaceae (ISO 21528-2:2004), *Escherichia coli* (ISO 16649-2:2001), *B. cereus* (ISO 7932:1987), *C. perfringens* (ISO 7937:1997), *Campylobacter* spp (Vidas Campylobacter (CAM), bioMérieux), *Staphylococcus aureus* (ISO 6888-1:1999) and *Listeria monocytogenes* (Vidas Listeria monocytogenes II, bioMérieux). Water samples were processed according to standard membrane filter procedures for enumeration of culturable micro-organisms (ISO 6222:1999), *E. coli* (ISO 9308-1:2000), coliform bacteria (ISO 9308-1:2000), *Pseudomonas aeruginosa* (ISO 16266:2006) and faecal streptococci (ISO 7899/2:1984), and detection and enumeration of the spores of sulfite-reducing anaerobes (Clostridia) (ISO 6461-2:1986). No virological analysis was performed on food and water samples because samples were exhausted in the bacteriological analysis.

RESULTS

During this two-year surveillance period, a total of four norovirus outbreaks were recorded. Questionnaires were distributed to a total of 618 soldiers from the affected military settings. A summary of the epidemiological and clinical data retrieved from the questionnaires, as well as the results from microbiological analysis, is provided in Table 1. The epidemic curves are presented in Figure 1.

Outbreak 1, October 2015

This outbreak occurred in stationed military personnel at a base in the north of Portugal. From a total of 90 soldiers at the base, 36 met the definition of probable AGI case (attack rate of 40%). Twenty-two soldiers had to receive medical treatment at the military hospital, given the severity of dehydration which required intravenous fluid therapy. Soldiers with AGI reported having shared the same sanitary facility, suggesting that fomites within this site might have played a role in the norovirus disease spread. Food items and water from the unit were tested and showed negative bacteriological results. The only stool sample that was obtained from this outbreak tested negative for enteropathogenic bacteria but positive for norovirus GII.17.

Outbreak 2, January 2016

This outbreak occurred in stationed military personnel at a base in one of the islands of Azores. From a total of 50 soldiers, 20 met the definition of probable AGI case (attack rate of 38%). No soldier required hospital treatment. Since all soldiers reported to have eaten in the same canteen, the outbreak was traced back to the preparation of the meal. Moreover, a food handler got sick with diarrhoea the day before preparing lunch for the base soldiers. The meal included a salad that 18 soldiers ate, all developing AGI symptoms within 24 hours. Interestingly, one of these soldiers was a vegetarian who has only eaten this salad. No food or water samples were obtained for microbiological analysis. Only five stools were obtained from this outbreak. They all tested negative for enteropathogenic bacteria but positive for norovirus GII.Pe-GII.4 Sydney.

Table 1 Summary of epidemiological/clinical data retrieved from the questionnaires and results of the microbiological analysis of the four norovirus outbreaks that occurred in the Portuguese Army, 2015–2017

	Outbreak 1	Outbreak 2	Outbreak 3	Outbreak 4
Date	October 2015	January 2016	November 2016	January 2017
Norovirus strain	GII.17	GII.Pe-GII.4	GII.P2-GII.2	GII.P16-GII.2
Region of Portugal (mainland) and islands	North	Azores	Centre	Centre
Military setting	Base	Base	Base	Exercise
Number of probable AGI cases/total in setting	36/90	20/50	26/394	17/84
Attack rates (%)	40	38	7	20
Duration of outbreak	3	4	4	3
Symptoms, n (%)				
Diarrhoea	10 (28)	16 (80)	15 (58)	10 (59)
Fever	6 (17)	6 (30)	5 (19)	4 (24)
Vomiting	23 (64)	18 (88)	17 (65)	16 (94)
Nausea	25 (69)	11 (55)	22 (85)	12 (71)
Abdominal pain	29 (71)	18 (88)	17 (65)	10 (59)
Number of cases admitted at the hospital, n (%)	22 (61)	0 (0)	2 (8)	6 (35)
Number of stool samples collected	1	5	17	4
Number of stool samples positive for norovirus by RT-PCR	1	5	11	3
Food and water bacterial investigation	Negative	Not done	Negative	Negative

AGI, acute gastroenteritis illness; RT-PCR, real-time PCR.

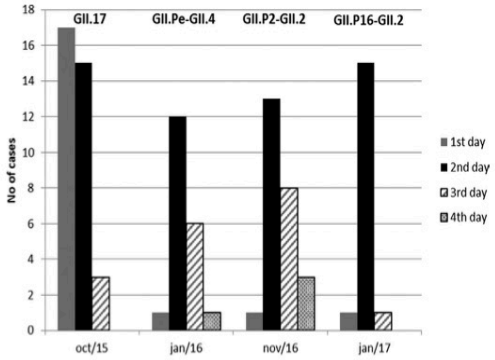

Figure 1 Epidemic curves of outbreak 1 (October 2015, norovirus GII.17), outbreak 2 (January 2016, norovirus GII.Pe-GII.4), outbreak 3 (November 2016, norovirus GII.P2-GII.2) and outbreak 4 (January 2017, GII.P16-GII.2).

Outbreak 3, November 2016

This outbreak occurred in stationed military personnel at a base with four platoons in the centre of Portugal. From a total of 394 soldiers at the base, 26 met the definition of probable AGI case (attack rate of 6.6%), all of whom belong to three of the four platoons of the base. Two soldiers had to receive medical treatment at the military hospital given the severity of symptoms. Since all soldiers from the four platoons reported to have eaten at the only canteen of the base, and AGI has occurred in soldiers from only three platoons, the point of origin was likely not related to the mess. Of note, AGI diseased soldiers reported having went to the same base pub the night before the onset of symptoms. No soldier from the fourth platoon went to that pub. Hence, it is plausible that this pub could have been the point of origin of the outbreak. Food items and water served at the canteen were tested for enteropathogenic bacteria, and one food item showed the presence of *E. coli* above 10^2 colony-forming unit/g. Only 17 stools were obtained from this outbreak. They all tested negative for enteropathogenic bacteria, but 11 showed to be positive for norovirus GII.P2-GII.2.

Outbreak 4, January 2017

This outbreak occurred during a military exercise in the centre of Portugal. From a total of 84 soldiers at the base, 17 met the definition of probable AGI case (attack rate of 20%). Six soldiers had to receive medical treatment at the military hospital given the severity of symptoms. Food items and water from the unit were tested and showed negative bacteriological results. Only four stool samples were obtained from this outbreak. They all tested negative for enteropathogenic bacteria, but three showed to be positive for norovirus GII.P16-GII.2.

DISCUSSION

Between October 2015 and October 2017, four norovirus outbreaks were detected by the surveillance system of reportable acute gastrointestinal illness in the Portuguese Army, under the coordination of the Laboratory of Bromatology and Biological Defence (LBBD). This study describes the laboratory and epidemiological data of these outbreaks, providing a thorough analysis of the circulation and impact of norovirus disease in the Portuguese Army.

From the 618 enquired soldiers, 99 met the definition of probable AGI case. Twenty-seven of these soldiers provided a stool sample, of which 20 were positive for norovirus by RT-PCR. Of note, 30 soldiers had to receive medical treatment due to the severity of symptoms. Comparing the data collected in this study with previous norovirus outbreak ad hoc reports from our team, it seems norovirus infections have been a constant presence in the Portuguese Army.[5 6] Corrective measures have been implemented after these outbreaks,[5 6] such as the distribution of mineral water during military exercises and the treatment of groundwater in Army units. Moreover, in the presence of a gastroenteritis outbreak, basic measures such as hand-washing practices, disinfection of premises and isolation of food handlers are routinely enforced as standard procedure. Despite all these measures, the four outbreaks described here have still occurred.

The present surveillance also shows the genotypic diversity of norovirus variants circulating in the Portuguese Army. In fact, the four norovirus outbreaks were caused by different strains, namely GII.17 (outbreak 1 in October of 2015), GII.Pe-GII.4 Sydney (outbreak 2 in January 2016), GII.P2-GII.2 (outbreak 3 in November 2016) and GII.P16-GII.2 (outbreak 4 in January 2017). All these four variants have been reported in European countries in the same period.[11] In fact, GII.17 strains emerged globally in winter 2014–2015 and were widely detected among most European countries between 2015 and 2016.[11] GII.4 Sydney started to be the dominant GII.4 variant in 2012–2013 and has circulated primarily as a recombinant, with GII.Pe (GII.Pe-GII.4 Sydney) being the predominant variant until winter 2015 when GII.P16 variant (GII.P16-GII.4 Sydney) emerged.[11 12] Non-GII.4 strains have also been circulating in the European territory, contributing to the norovirus disease burden, such as GII.2 which has been predominantly circulated and associated with GII.P2 (GII.P2-GII.2).[11 12] A GII.2 variant with a novel GII.P16 polymerase (GII.P16-GII2) has emerged in late 2016 and has been associated with a high number of noroviruses infections since then.[11 12]

With regard to outbreak 1, although no definite origin was identified, it was possible to know that the AGI soldiers had shared the same sanitary facility, suggesting that this site might have played a role in the norovirus transmission. The contamination of fomites by norovirus aerosolisation has been documented in a variety of settings[13] and might also have taken place here. Caution must be taken when pointing to the cause of this outbreak since only one stool sample was available for analysis, not allowing for a definitive diagnosis of the cause of outbreak.

Outbreak 2 was traced back to the consumption of salad prepared by a food handler who had gotten gastroenteritis the day before the onset of the outbreak. A vegetarian soldier who has exclusively eaten the salad and developed AGI strengthens this hypothesis. Unhygienic manipulation by both symptomatic and asymptomatic food handlers has been linked with norovirus transmission.[14 15] The investigation of outbreak 3 presented several drawbacks that hampered the identification of an origin. As an entire platoon was also eating from the same mess and did not show any case of disease, it seemed tempting to exclude the mess as the point of origin of this outbreak. The only common event of all AGI soldiers was going to an Army base pub. Whether this was the point of origin remains to be confirmed. With regard to outbreak 4, no epidemiological data were available to suggest a point of origin for the norovirus outbreak. However, the norovirus strain responsible for the disease was identical to the one found in France in the winter of 2016–2017 during the Christmas festivities.[16] Since this outbreak occurred in early January, it is tempting to speculate that a transboundary origin from France might have occurred since there there is a strong traditional Christmas travelling of Portuguese emigrants

in France back to Portugal to reunite with their families during the festivities.

The control and mitigation of norovirus infection in military settings should be based on strict hygienic measures as there are currently no vaccines or antivirals for the treatment of norovirus disease.[17]

In conclusion, the present report shows that noroviruses have a major role in gastroenteritis outbreaks in the Portuguese Army, both in stationed and in an exercise context. However, other causes of gastroenteritis outside the context of outbreaks may also play an important role and deserve to be studied in the military forces. Since physical disability due to norovirus disease in soldiers interferes with induction and training schedules, this results in a high number of non-effective military personnel, diminishing operational effectiveness and impeding force readiness.

Acknowledgements The authors would like to acknowledge Unidade de Tecnologia e Inovação, Departamento de Genética Humana, Instituto Nacional de Saúde Dr Ricardo Jorge, Lisbon, Portugal, for Sanger sequencing.

Contributors AL-J, JRM, RdS, MO, CS, CP-G and MSJN equally contributed to the manuscript.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient consent Not required.

Provenance and peer review Not commissioned; externally peer reviewed.

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4. Simultaneous norovirus outbreak in three Portuguese Army bases in Lisbon region, December 2017

This study describes epidemiological, clinical and laboratorial investigations of a multicenter gastroenteritis outbreak that occurred simultaneous in three Portuguese Army Units of Lisbon region, in December 2017. Although the Units are localized several kilometers apart they had a common food supplier. The three simultaneous acute gastroenteritis outbreaks affected 31 soldiers from a total of 874 stationed at the three units who had consumed meals in the military premises 72h prior the outbreak onset. From the 11 stool samples obtained from the affected soldiers all were negative for the common enteropathogenic bacteria but tested positive for norovirus. Sequence analysis identified the recombinant norovirus GII.P16-GII.4 Sydney in all positive samples with 100% nucleotide sequence identity among them. This strain was the most likely cause of the multicenter outbreak. Overall, results are suggestive of a common source of infection plausibly related to the food supplying chain.

The content of this section was published in the following paper:

Lopes-João A, Mesquita JR, de Sousa R, Oleastro M, Penha-Gonçalves C, Nascimento MS. (2019). Simultaneous norovirus outbreak in three Portuguese Army bases in the Lisbon region, December 2017. J R Army Med Corps. **0**:1-4.

Simultaneous norovirus outbreak in three Portuguese army bases in the Lisbon region, December 2017

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Received 13 May 2019
Revised 17 June 2019
Accepted 18 June 2019

To cite: Lopes-João A, Mesquita JR, de Sousa R, *et al*. *J R Army Med Corps* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/jramc-2019-001242

ABSTRACT

Introduction Norovirus outbreaks frequently occur in communities and institutional settings acquiring a particular significance in armed forces where prompt reporting is critical. Here we describe the epidemiological, clinical and laboratorial investigation of a multicentre gastroenteritis outbreak that was detected simultaneously in three Portuguese army units with a common food supplier, Lisbon region, between 5 and 6 December 2017.

Methods Questionnaires were distributed to all soldiers stationed in the three affected army units, and stool specimens were collected from soldiers with acute gastrointestinal illness. Stool specimens were tested for common enteropathogenic bacteria by standard methods and screened for a panel of enteric viruses using a multiplex real-time PCR assay. Food samples were also collected for microbiological analysis. Positive stool specimens for norovirus were further genotyped.

Results The three simultaneous acute gastroenteritis outbreaks affected a 31 (3.5%) soldiers from a total of 874 stationed at the three units and lasted for 2 days. No secondary cases were reported. Stool specimens (N=11) were negative for all studied enteropathogenic agents but tested positive for norovirus. The recombinant norovirus GII.P16-GII.4 Sydney was identified in all positive samples with 100% identity.

Conclusions The results are suggestive of a common source of infection plausibly related to the food supplying chain. Although centralisation of food supplying in the army has economic advantages, it may contribute to the multifocal occurrence of outbreaks. A rapid intervention is key in the mitigation of outbreak consequences and in reducing secondary transmission.

Key messages

- Norovirus GII.P16-GII.4 Sydney was responsible for a gastroenteritis outbreak occurring simultaneously in three Portuguese army units.
- Centralisation of food supplying likely contributed to the multifocal outbreak.
- Rapid implementation of control measures contributed to the short outbreak duration and absence of secondary cases.
- A robust food and waterborne outbreak surveillance system is key in dissuading intentional contaminations of the food chain in the army.

INTRODUCTION

Norovirus gastroenteritis outbreaks frequently occurs in communities and institutional settings acquiring a particular significance in armed forces where prompt reporting is critical.[1] Gastrointestinal infections in the US Armed Forces have consistently been ranked among the most frequently reported diseases and non-battle injuries,[2] with noroviruses being highlighted as the primary aetiology of both sporadic cases and outbreaks of acute gastroenteritis.[3 4] Norovirus outbreaks have also been described in the Portuguese army either in stationed forces or in exercise context causing considerable morbidity with impact in the force readiness.[5–7]

Food, water and food handlers have been frequently pointed as the source of norovirus outbreaks within the military.[7–9] Uncooked foods, like lettuce salads, have been implicated or at least suspected in norovirus outbreaks in armed forces.[10–12] As close living conditions prevail in the military basements, secondary transmission is considered an important factor in further amplifying the number of cases and duration time of the outbreaks.[13]

The present study reports the investigation of a simultaneous gastroenteritis outbreak that was detected in three Portuguese army units from Lisbon region that are distanced several kilometres apart, in December 2017. It affected 31 individuals among the 874 soldiers stationed in the three units who had consumed meals in the military premises 72 hours prior the outbreak onset.

The outbreak was caused by GII.P16-GII.4 Sydney norovirus recombinant strain, found in stools of affected soldiers from the three units. This genotype was first described in late 2014 and has been found circulating worldwide,[14] being considered highly transmissible with a pandemic potential.[15] In a recent study conducted in the German military health system, this strain was found to be the causative agent for 16% of all norovirus outbreaks during the 2016–2017 winter season.[16]

In the current paper, the epidemiological, clinical and laboratorial findings of this multicentre norovirus outbreak are described.

MATERIALS AND METHODS

Case definition

A norovirus probable acute gastrointestinal illness (AGI) case was defined as a soldier who experienced acute diarrhoea (three or more episodes of loose stools in a 24-hours period) or presented vomiting with at least one of the following symptoms: one or

more episodes of loose stools in a 24-hours period, abdominal cramps, headache, muscle aches or fever.[7]

Epidemiological data and sample collection

On 5 December 2017, a simultaneous gastroenteritis outbreak was detected by patient attendance at medical centres of three army units that following a standard operating procedure immediately notified the Army Health Directorate (AHD). The Veterinary Unit of the AHD was then asked to manage the outbreak at the epidemiological and laboratory level, including analysis of clinical and food samples. The three army units are located in the Lisbon region but several kilometres apart (35 km between units A and B, 15 km between units A and C and 25 km between units B and C).

Relevant epidemiological information focused on clinical signs, and ingested food items were collected from soldiers of the three units through a standard questionnaire handed to both AGI affected and non-affected soldiers. Data included demographic information, clinical signs and symptoms, date of onset and consumed food items from the previous 3 days. Out of the 31 affected individuals that met the definition of probable AGI case, only 12 (39%) volunteered to participate in this study by allowing stool sampling (six from unit A, two from unit B and four from unit C).

Microbiological investigation of stools

Stools were analysed for common enteropathogenic bacteria, namely, *Shigella*, *Salmonella*, *Campylobacter*, *Vibrio*, *Bacillus cereus* using standard coproculture methods. Counting of *Clostridium perfringens* spores was also performed. Stools were also screened for a panel of enteric viruses, namely, norovirus, astrovirus, rotavirus, adenovirus and sapovirus, using a commercial multiplex real-time PCR (FTD Viral Gastroenteritis, Fast Track Diagnostics, Luxembourg) according to manufacturer's instructions. Noroviruses were genotyped based on ORF1 and ORF2 sequences as described previously.[17 18] Genotype assignment was performed using the automated genotyping tool from Noronet platform.[19]

Microbiological investigation of food

Food samples from meals (soups, main dishes and lettuce salads) served in the affected units, in the 24 hours prior the detection of the AGI cases were collected and tested for *Salmonella* spp (Vidas Salmonella (SLM), bioMerieux), Enterobacteriacea (ISO 21528-2:2004), *Escherichia coli* (ISO 16649-2:2001), *B. cereus* (ISO 7932:1987), *Clostridium perfringens* (ISO 7937:1997), *Campylobacter* spp (Vidas *Campylobacter* (CAM), bioMerieux), Coagulase positive *Staphylococcus* (ISO 6888-1:1999) and *Listeria monocytogenes* (Vidas *L. monocytogenes* II, bioMerieux). Due to exhaustion of samples in the bacteriological analysis, no virological detection on food items was performed.

RESULTS

A summary of the epidemiological and clinical data obtained from the questionnaires as well as the results from microbiological analysis of stools is provided in table 1. The first case was observed at 17:30 of 5 December and the last at 20:00 of 6 December 2017. The attack rates on the three army units varied from 3.3% to 5%, with diarrhoea being the most observed symptom, followed by vomiting. From the 12 stool specimens collected from the soldiers with AGI, 11 showed positive to norovirus. No enteropathogenic bacteria were found in stools that also showed negative for astrovirus, rotavirus, adenovirus and sapovirus. The DNA sequences retrieved from all norovirus-positive samples showed to be identical (100% identity in both ORF1 and ORF2 regions) and were classified as norovirus GII.P16-GII.4 Sydney.

Table 1 Summary of epidemiological/clinical data retrieved from the questionnaires and results from microbiological analysis of stools of the norovirus outbreaks that occurred in three units of the Portuguese army

	Army unit A	Army unit B	Army unit C
First case reported	17:30; 5 December 2017	19:30; 5 December 2017	23:00; 5 December 2017
Last case reported	08:00; 6 December 2017	6:50; 6 December 2017	20:00; 6 December 2017
No. probable AGI cases/total in setting	13/414	10/300	8/160
Estimated attack rates	3.3%	3.5%	5%
Mean ages of affected soldiers	24	26	25
Symptoms, n (%)			
Diarrhoea	11 (84%)	8 (80%)	7 (88%)
Vomiting	11 (84%)	5 (50%)	5 (63%)
Nausea	8 (62%)	5 (50%)	5 (63%)
Abdominal pain	7 (54%)	4 (40%)	3 (38%)
Fever	7 (54%)	1 (10%)	2 (25%)
No. cases treated at medical centre, n (%)	4 (31%)	0 (0%)	3 (38%)
No. stools collected samples	6	2	4
No. stool samples positive for norovirus by reverse transcription-PCR	5	2	4
Norovirus strain	GII.P16-GII.4 Sydney	GII.P16-GII.4 Sydney	GII.P16-GII.4 Sydney

AGI, acute gastroenteritis illness

The microbiological results of the food items from both lunch and dinners of 4 and 5 December 2017 are shown in table 2. The majority of tested meals were of acceptable bacteriological quality with the exception of the lettuce salad served on unit B at lunch, and the main dish served on unit A at dinner, on 5 December.

Although the three army units were several kilometres apart and no troop movements occurred among them, their food supplier was the same. The three units offered the same warm meals on 4 and 5 December 2017 were the same. Unit B receives meals prepared in kitchen from unit A, while the unit C prepared its own meals on site. Raw vegetables (lettuce salad) were distributed directly from the same market supplier to the kitchens of units A and C. Due to incomplete information on food consumption by the affected individuals, a risk analysis for the consumed items was impossible to perform. Moreover, when questioned for any recent or ongoing gastrointestinal illness, food handlers and food suppliers reported not having been sick.

DISCUSSION

The present study reports the epidemiological and microbiological analysis of a multicentre gastroenteritis outbreak that occurred in three units of the Portuguese Army forces in December 2017. The recombinant norovirus GII-P16-GII.4 Sydney was the most likely cause of the outbreak.

This outbreak revealed an interesting epidemiological profile, namely: (1) it affected three army units simultaneously; (2) all cases occurred in a 2-day period and (3) estimated attack rates were relatively low for a norovirus outbreak and were similar in

Table 2 Microbiological results of the food items investigated during the outbreak

		Unit A		Unit B		Unit C	
Day	Meal	Food item	Bacteriological result	Food item	Bacteriological result	Food item	Bacteriological result
4 December	Lunch	Main dish and soup	Acceptable	No sample	–	Main dish and soup	Acceptable
	Dinner	Main dish	Acceptable	No sample	–	Main dish and soup	Acceptable
5 December	Lunch	No sample	–	Main dish and soup Lettuce salad	Acceptable Enterobacteria 1.5×10^5 CFU/g	Main dish and soup	Acceptable
	Dinner	Main dish	*E.coli* $4{,}3\times10^4$ CFU/g and *Bacillus cereus* 2.5×10^5 CFU/g	Main dish and soup	Acceptable	Main dish and soup	Acceptable

CFU, colony-forming unit.

the three units. This profile indicates that norovirus was introduced at the same moment in the three units and is suggestive of a common source of infection. The simultaneous occurrence of the outbreak in army units A and B could be easily explained by the centralization of the kitchen services. The use of the same kitchen is a consequence of a logistical rational approach for a coherent use of resources. This approach in the logistics of meal preparation can potentiate the dissemination of a gastroenteritis agent and favour the occurrence of multifocal outbreak phenomena. Nevertheless, the occurrence of disease in unit C, where meals were independently prepared by a different team and in a different setting clearly point the origin of the outbreak to a common food item. The lettuce salad was suspected since it was the only raw item to be consumed. Moreover, the lettuce was provided by the same supplier and had been served in the 2 days prior the outbreak onset in the three units. The only lettuce sample sent for analysis (served at lunch of 5 December 2017) presented high counts of enterobacteria (1.5×10^5 colony-forming unit/g); hence, it is tempting to hypothesise that viral contamination might have come from this common source. In fact, lettuce salad has been frequently implicated in the origin of norovirus outbreaks in military settings.[7 10 11]

This multicentre norovirus outbreak was also characterised by a short 2-day period of duration and absence of secondary cases. This may be explained by the rapid implementation of control measures both by food and clinical sectors of the army forces that included sanitisation of facilities with bleach, reinforcement of hands hygiene and strengthening disinfection procedures of salads and fresh fruits. These measures may have contributed to the mitigation of secondary person-to-person transmission and absence of secondary cases. Curiously, the attack rates were similar in the three units and were low when compared with the mean attack rate observed in previous norovirus outbreaks in the Portuguese army.[5–7]

However, some limitations should be noted in this study. It was not possible to identify the exact source of the outbreak or to perform a risk analysis on the food items consumption since food samples were not preserved for analysis for the required time and many questionnaires were not duly filled by the soldiers. Additionally, only 12 stool samples were obtained from a total of 31 soldiers with AGI as they are asked to provide samples voluntarily.

This outbreak in multiple military sites illustrates the increased risks of a logistic system using common suppliers and centralised meal production. Under prompt medical assistance, AGI usually represents a moderate and transitory individual health threat but in the context military units may have a significant impact in operational capability and force readiness. This work highlights the benefits of a gastrointestinal outbreak surveillance system as a tool to enable ready medical services response, detection the causative agent and its origin as well as fast implementation of control measures. Furthermore, the possibility of multicentre outbreaks draws attention for the relevance of a robust food and waterborne outbreak surveillance system in dissuading intentional contaminations of the army food supply chain.

Acknowledgements The authors would like to acknowledge Unidade de Tecnologia e Inovação, Departamento de Genética Humana, Instituto Nacional de Saúde Dr. Ricardo Jorge, Lisbon, Portugal for Sanger sequencing.

Contributors All authors have equally contributed to the manuscript.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests None declared.

Patient consent for publication Not required.

Provenance and peer review Not commissioned; internally peer reviewed.

Data availability statement All data relevant to the study are included in the article or uploaded as supplementary information.

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5. Characterization of the norovirus outbreaks reported in the Portuguese Army

A global analysis of the epidemiology, transmission routes, genotypes involved and trends of all the noroviruses outbreaks that occurred in the Portuguese Army during the five-year period of this study is presented here. The role of norovirus as a cause of sporadic gastroenteritis in the military personnel is also discussed.

5.1. Summary of the norovirus outbreaks

During the five-year (2013-2017) study period, 14 outbreaks of gastroenteritis (Figure 7) that affected a total of 410 military (Figure 8) were registered and investigated in the Portuguese Army.

Noroviruses were responsible for eight (57%) of these outbreaks, while five had a bacteria or bacterial toxin origin and in one it was not possible to identify the causing agent (Figure 7). Two norovirus outbreaks were registered in 2013, 2015, 2016 and 2017. No outbreak was reported to the BBDL, in 2014.

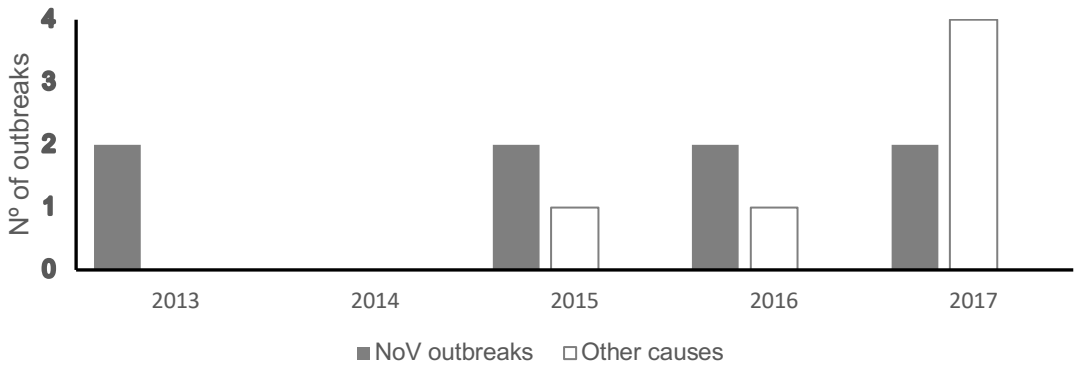

Figure 8. Number of gastroenteritis outbreaks caused by norovirus *versus* other causes, reported in the Portuguese Army, 2013-2017.

Noroviruses were responsible for 210 (51%) cases accounting for the majority case numbers (Figure 8).

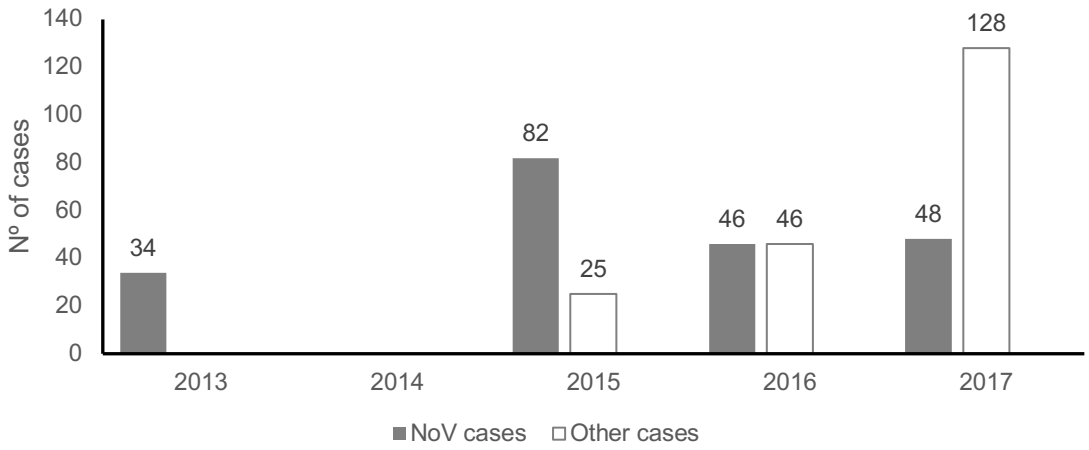

Figure 9. Number of cases reported in the 14 gastroenteritis outbreaks, caused by noroviruses *versus* other causes, reported in the Portuguese Army, 2013-2017.

The eight outbreaks occurred in the North, Center, Lisbon region and in the Azores. Based on the data of this five-year period it seems that the number of outbreaks of norovirus is rather constant in the Portuguese Army, with a mean number of 1.6 outbreaks per year and throughout the Portuguese territory. Although no norovirus outbreak was notified to the BBDL in 2014, at least two outbreaks were registered in the other years of the study, namely in 2013, 2015, 2016 and 2017, leading to the conclusion that noroviruses were a major cause of gastroenteritis among the Portuguese Army personnel.

The number of cases, per outbreak, caused by noroviruses varied from 15 to 46 while the outbreaks caused by bacteria or bacterial toxins ranged from 10 to 85 cases (Figure 9).

A seasonal pattern was observed in the norovirus outbreaks. Half occurred in winter months, three in the autumn and only one in the spring (Figure 9). During the summer months, there was no report of norovirus outbreaks.

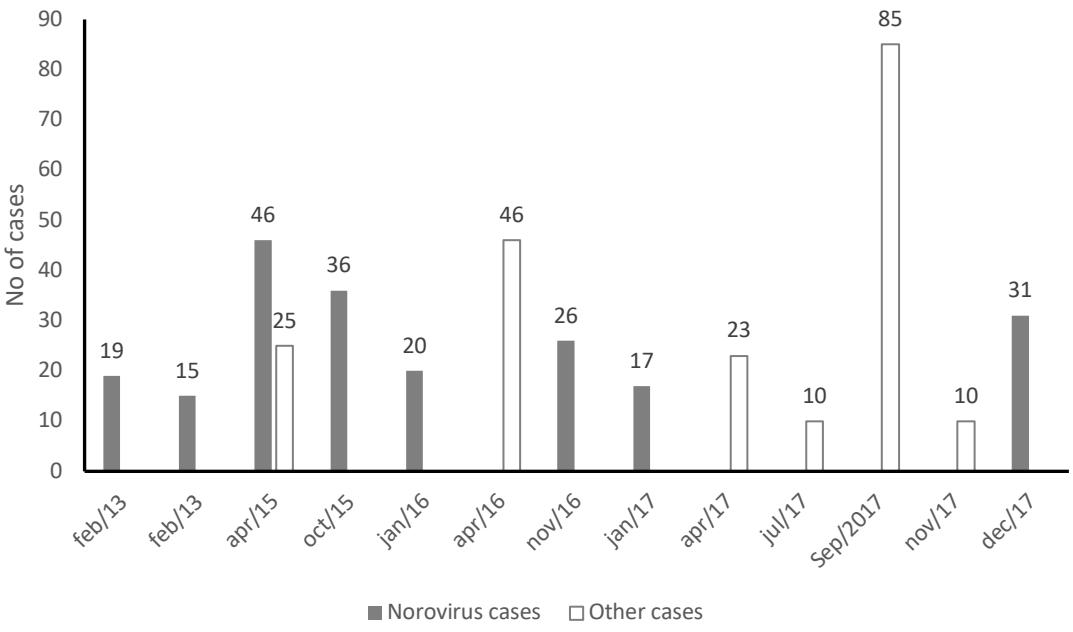

Figure 10. Number of cases of gastroenteritis caused by noroviruses *versus* other causes and the temporal distribution of the outbreaks, reported in the Portuguese Army, 2013-2017.

Concerning the attack rate, it ranged from 4% to 40% in outbreaks caused by norovirus and from 2.4% to 50.5% in the outbreaks caused by bacteria and bacteria toxins (Figure 10). The higher attack rates of norovirus outbreaks, 40%, 38% and 20% were associated to person-to-person transmission (October 2015 outbreak) and to a food handler (January 2016 and January 2017 outbreaks), respectively. Higher attack rates have been traditionally reported in food- and waterborne outbreaks when compared to person-to-person transmission (Matthews et al. 2012). Differences in implicated foods, susceptibility of the host to norovirus infection and pathogenicity of norovirus strains may influence the attack rate in foodborne outbreaks (Noda et al. 2008).

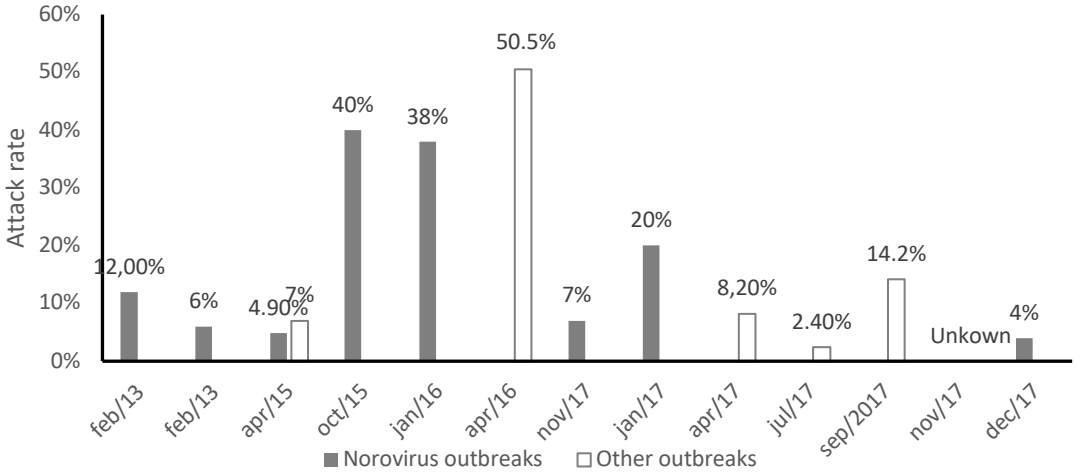

Figure 11. Estimated attack rates of the 14 gastroenteritis outbreaks, reported in the Portuguese Army, 2013-2017.

The clinical details of the eight norovirus outbreaks are summarized in Table 6. Outbreaks lasted from two to nine days at the most, with a mean duration time of four and a half days. The symptoms reported from the sick soldiers included, vomiting in 79.3% of the cases, nausea in 69.1%, diarrhea in 64.4% and fever in 21.3%. About 20.6% of the soldiers with acute gastroenteritis had to receive parenteral fluid therapy at the health centers or even at the hospital in order to replace electrolyte and fluid loss lost in bouts of diarrhea and vomiting.

Table 6. Clinical details of the norovirus outbreaks reported in the Portuguese Army, 2013-2017

Outbreaks Date	Attack rate (%)	Duration (days)	Vomiting (%)	Nausea (%)	Diarrhea (%)	Fever (%)	Parenteral Fluid Therapy (%)
Feb 2013_1	12 (19/160)	7	85	85	45	25	20
Feb 2013_2	6 (15/250)	9	66	53	40	13	0
Apr 2015	4.9 (46/938)	5	81	65	85	10	8
Oct 2015	40 (36/90)	3	64	69	28	17	22
Jan 2016	38 (20/50)	4	88	55	80	30	0
Nov 2016	7 (26/394)	4	65	85	58	19	8
Jan 2017	20 (17/84)	3	94	71	59	24	35
Dec 2017	4 (31/874)	2	71	70	87	32	31
Mean value	16.6	4.5	79.3	69.1	64.4	21.3	20.6

The annual incidence rate of cases of norovirus gastroenteritis, in outbreak context, ranged from 1.3 to a maximum of 5.9 per 1000 active military with an overall mean of 2.9 cases per 1000 (Table 7). For the calculation of the incidence rates only the military personnel in duty were considered as these personnel is responsible for operational activity and force readiness. The absenteeism due to gastrointestinal illness of on duty personnel has a direct impact in the daily routines of the Military Units and the accomplishment of the mission.

Table 7. Annual incidence rate of norovirus gastroenteritis in the active Army military, 2013-2017

Year	Number of cases	Army active (on duty) personnel	Incidence rate
2013	20*	17637	1.3 per 1000
2014	-	15071	-
2015	82	14004	5.9 per 1000
2016	46	13351	3.4 per 1000
2017	48	12230	3.9 per 1000

* Number of cases in 2013 are less than the 34 mentioned in table 6, because only the military on duty were considered here. The data from Army active personnel was withdrawed from "Desafios da Estratégia Militar Nacional" in Revista Militar nº2/3 fevereiro/março 2018 pp (135-184).

5.2. Modes of transmission and key features of the Portuguese Army norovirus outbreaks

Several modes of norovirus transmission were suspected or suggested in these eight norovirus outbreaks of the Portuguese Army (Table 8). Five (63%) were likely foodborne (including food handlers) or waterborne and three (37%) had a predominant person-to-person transmission (Table 7). The origin of the outbreak was difficult to confirm in most cases but in two outbreaks (January 2016 and January 2017) diseased food handlers were the likely cause of the outbreaks, as in both of them food handlers got sick with gastroenteritis the day before of the outbreaks.

Two of the outbreaks of the Portuguese Army were not linked to the Army base cuisines. The epidemiological data linked one of them to the utilization of sanitary facilities (October 2015 outbreak), attributing to fomites within this place a role in norovirus spread and the other the visit of a base pub (November 2016 outbreak), the most probable point of origin of the outbreak.

But overall it is important to highlight that seven norovirus outbreaks occurred in Army active personnel either stationed in military units or in military exercises, impacting the daily routines and the operational activities.

Table 8. Point of origin, main transmission way and key features of the norovirus outbreaks, reported in the Portuguese Army, 2013-2017

Outbreaks date	Point of origin suspected	Way(s) of transmission	Key features of the outbreak
Feb 2013_1 [a]	Water from a creek	Water borne, and secondary person-to-person transmission	Occurred during a military exercise, norovirus was identified in stools
Feb 2013_2	Possible food as *E.coli* (fecal indicator) was detected in some food items	Direct transmission through persons	Occurred in a Military nursing home; the nurse who attended the first cases developed the same symptoms and norovirus was identified on his stools
Apr 2015 [b]	Ground water	Water borne	Occurred in an Army base
Oct 2015 [c]	Sanitary facilities	Direct transmission through persons or fomites	Occurred in an Army base; a military got sick after cleaning the vomit of a soldier with gastroenteritis; no link with the food sector of the Unit
Jan 2016 [c]	Food handler	Foodborne or cutlery manipulated by the food handler	Occurred in an Army base; only the soldiers that attended the canteen of the sick food handler developed symptoms; a person who only ate a salad prepared by this food handler got also sick
Nov 2016 [c]	Base Pub	Direct transmission through persons or fomites	Occurred in an Army base; affected three of the four platoons
Jan 2017 [c]	Food handler	Foodborne or cutlery manipulated by the food handler	Occurred during a military exercise
Dec 2017 [d]	Salad	Foodborne	Occurred simultaneously in three Army bases;

[a] outbreak described in article 1;
[b] outbreak described in article 2;
[c] outbreak described in article 3;
[d] outbreak described in article 4.

Also, of note, was the outbreak that occurred in a care facility center of the Portuguese Armed Forces in February 2013. This outbreak affected 15 persons. The first seven persons got sick in day one after eating different food items in the canteen. New cases kept appearing in the following days. Two days after the onset of the outbreak the nurse (who had no meal in the canteen) got sick with the same symptoms, that were predominantly vomit (n=10; 66%) and diarrhea (n=5; 40%). No pathogenic bacteria were found in the 11 analyzed food items, but two had fecal indicator (*Escherichia coli*) above the acceptability level (100cfu/g). The nurse stool sample tested positive for norovirus GII.4 Sydney 2012. Although it is not possible to confirm that the gastroenteritis outbreak was due to norovirus, epidemiological data strongly suggest that this virus could have been the culprit. It is plausible that the event had begun in the facility canteen since some food items presented fecal contamination and that subsequently person-to-person transmission lead to the nurse infection.

5.3. Sporadic norovirus gastroenteritis in the Portuguese Army

The role of norovirus as a cause of sporadic gastroenteritis in the military personnel was also evaluated during 2013–2017. Unfortunately, only 15 samples were collected in the context of sporadic acute gastrointestinal illness in health care centers and sent to the BBDL. In fact, healthy adults with acute gastroenteritis do not usually go to the health care centers for treatment and likewise health professionals do not usually take stool samples to do the laboratory diagnosis of single cases of gastroenteritis because most immunocompetent patients recover in few days. However, from the 15 cases of sporadic gastroenteritis, four (26,6%) were found positive for norovirus. Norovirus GII was identified in all the stools but in one a co-infection with norovirus GI was detected. The genotyping of the noroviruses, was not performed since it has little epidemiological relevance in sporadic cases. Although the low number of cases studied does not allow to evaluate the incidence of sporadic norovirus associated gastroenteritis in the Portuguese Army, it is tempting to assume that noroviruses are also a frequent cause of sporadic cases of acute gastroenteritis. This is in accordance with other studies that identified norovirus as the primary etiology of both sporadic cases and outbreaks of acute gastroenteritis among trainees (Delacour et al. 2010; Brooks et al. 2018).

5.4. Norovirus genogroups/genotypes involved in the Portuguese Army outbreaks

Norovirus GII accounted for the majority of norovirus outbreaks, six in a total of eight (75%) and cases, 145 in a total of 210 (69%) whereas norovirus GI was responsible for two (25%) outbreaks and 65 (31%) cases (Table 9). The co-infection case observed in the first outbreak of February 2013 was counted has a GI case. Curiously, the GI outbreaks were suspected to be waterborne. In fact, norovirus GI are more often detected in waterborne outbreaks (Cheng et al. 2017; Lian et al. 2019). In the first outbreak of February 2013, that occurred during a military exercise, a co-infection with norovirus GI.3 and GII.4 was detected although in the majority of the stool samples only norovirus GI.3 was found. Military can be more exposed to uncontrolled spring water than general population, since in military exercise context soldiers may have access to untreated water that can be contaminated with stools, what could have happened in the outbreak of February 2013. The outbreak of April 2015 was also caused by norovirus GI and was associated with poorly treated ground water, that was supplied to the Army base canteens. Groundwater is an important source of drinking water but a deficient treatment can expose people to norovirus, as this agent is highly resistant to environmental degradation and long-term infectivity has been reported for groundwater (Katayama and Vinjé 2017; Lee et al. 2018).

Table 9. Number of cases and genogroups/genotypes involved in the norovirus outbreaks of the Portuguese Army, 2013-2017

Outbreaks date	Number of cases	Norovirus Genogroup/Genotype
Feb 2013_1	19	GI.3 co-infection GII.4 New Orleans 2009 (1 case)
Feb 2013_2	15	GII.4 Sydney 2012
Apr 2015	46	GI.9
Oct 2015	36	GII.17 Kawasaki 2014
Jan 2016	20	GII.Pe-GII.4 Sydney 2012
Nov 2016	26	GII.P2-GII.2 Hiroshima 2010
Jan 2017	17	GII.P16-GII.2 France
Dec 2017	31	GII.P16- GII.4 Sydney 2012

Norovirus GII was suspected of being the cause of the outbreak of 2013 registered in a military nursing home. This variant GII.4 Sydney 2012 was related to an increase in norovirus

activity (outbreaks and illness) in late 2012 and became the predominantly detected variant worldwide since 2012 (Van Beek et al. 2018).

Since 2016 recombinant variants of GII.4 Sydney 2012 predominated in the outbreaks of the Portuguese Army. This data is also in line with other European studies that state that norovirus GII.4 Sydney 2012 have been evolving through recombination rather than antigenic replacement (van Beek et al. 2018). Even the GII.17 strain, that predominated in China and Japan in 2015, did not fully replace the GII.4 strains in Europe (van Beek et al. 2018). Another interesting result was the detection of the new norovirus recombinant GII.P16-GII.2 France as the causative agent of the outbreak of January 2017. The detection of this strain in Portugal a few months after being described in France (Bidalot et al. 2017), shows how rapid the propagation of noroviruses can occur.

Remarkably each outbreak had a different genotype(s), highlighting the great genetic diversity of circulating norovirus in the Portuguese military.

IV. DISCUSSION

The overall objective of this thesis was to evaluate the impact of norovirus in outbreaks of foodborne acute gastroenteritis reported in the Portuguese Army. This work was supported by the implementation of a surveillance system that reported all the outbreaks of gastroenteritis to the BBDL during the five-year period, 2013-2017.

Our previous observations led us to suspect that about half of foodborne disease outbreaks occurred between 2006 and 2012 had a viral etiology, since no pathogenic bacteria or bacteria toxins were detected in the suspected food items (Lopes-João 2013). Hence, knowing that noroviruses were the leading cause of foodborne illnesses we implemented molecular diagnostic methods for the detection and characterization of norovirus, in stool samples of soldiers presenting acute gastrointestinal disease. The detection of norovirus in food items was not chosen since the reliable detection in food matrices remains a challenge, due to the low level of viral contamination and the heterogeneous distribution of viral particles in foods (Bosch et al. 2018). In addition, coprocultures for the common enteropathogenic bacteria became part of the routine of the BBDL, testing whenever an outbreak of gastrointestinal illness occurred. In connection with this study a gastrointestinal outbreak surveillance system was established in the Portuguese Army that supported the routine collection of stool samples from military personnel with acute gastroenteritis.

During this five-year study norovirus “emerged” as the leading etiologic agent of acute gastroenteritis in the Portuguese Army. Norovirus accounted for the majority of the reported gastroenteritis outbreaks (57%) and for the highest number of cases (51%) among all causes of gastroenteritis. Norovirus gastroenteritis has been singled out as a military concern for 20 years now (Mccarthy et al. 2000; Delacour et al. 2010; yap et al. 2012; Brooks et al. 2018). In fact, noroviruses have been shown to be a major cause of acute morbidity in military forces and the primary cause of both sporadic cases and outbreaks of acute gastroenteritis among military trainees (Mccarthy et al. 2000; Grotto et al. 2004; Delacour et al. 2010; Ahmed et al. 2012; Brooks et al. 2018). Norovirus has been identified as one of the top five etiologic agents of gastroenteritis among military, although a study placed it after *Campylobacter*, nontyphoidal *Salmonella*, *Giardia* and *Shigella* (Hill et al. 2017). Authors explain this contradiction by the fact that circa 98.8% of the cases of gastrointestinal illness remain without diagnostic and were considered nonspecific highlighting the need to improve diagnostic testing to further elucidate the prevalence of causative agents of gastroenteritis within the military personnel (Hill et al. 2017). A more recent study estimated that norovirus account for 85% of foodborne illness and alert for the tremendous burden of acute foodborne gastrointestinal disease and its five major causative pathogens (C*ampylobacter jejuni*, *Shigella spp*., *Salmonella enterica* non- typhoidal, STEC non-O157 and noroviruses) in non-deployed active personnel from the USA Army, with about 45600 annual cases of illnesses (Mullaney et al. 2019).

Norovirus are also a primary cause of sporadic cases of acute gastroenteritis among military (Delacour et al. 2010; Ahmed et al. 2012; Brooks et al. 2018) and in this work the role of norovirus in sporadic acute gastroenteritis in the Portuguese Army was also evaluated. Although only 15 cases of sporadic acute gastrointestinal illness were studied, norovirus was detected in four (26.6%). This limited number of cases did not allow concluding extrapolations on the relative weight of norovirus in sporadic gastroenteritis in the Portuguese Army. Nevertheless, it revealed that the systematic laboratory testing of norovirus helps in the etiological diagnosis of gastrointestinal infections otherwise classified as nonspecific.

Norovirus outbreaks in the Portuguese Army were caused by genogroups I and II, but GII outbreaks clearly outnumbered those caused by GI norovirus. Norovirus GII accounted for the majority of the outbreaks, six in a total of eight (75%) and about 2/3 of the cases, 145 in a total of 210 (69%). This is in accordance with reports that found that more than 70% of norovirus outbreaks are caused by genogroup II (Fankhauser et al. 2002; Patel et al. 2009; Vega et al. 2014; Lee et al. 2015).

The first gastroenteritis outbreak investigated in this study occurred during a military exercise in central region of Portugal in February 2013 (Lopes-João et al. 2015). It had a considerable impact on the operational effectiveness given the severity of symptoms presented by the soldiers, which also led us to suspect of norovirus as the primary cause of disease. No pathogenic bacteria were found in the stools of the sick soldiers but the virologic diagnosis revealed the presence not only of norovirus GI and GII but also of two other enteropathogenic viruses namely, astrovirus and sapovirus. Although astrovirus and sapovirus can alone cause acute gastroenteritis, these viruses are primarily linked to severe acute gastroenteritis in infants, young children, elderly and immunocompromised individuals (Iturriza-Gómara et al. 2009; Lee et al. 2012; Oka et al. 2015). For this reason and given the severity of symptoms presented by this healthy young group of soldiers we suggested that norovirus was the probable cause of the outbreak. One of the soldiers was negative for norovirus but his stools presented both astrovirus and sapovirus, which could have resulted in an increased intestinal damage explaining the severe symptoms. The origin of this outbreak remains uncertain, since food and water were not analyzed for the presence of viruses, although creek water, used for drinking, was the most likely origin of this outbreak. Having detected norovirus GI.3 in all except one of the studied soldiers strengthens the hypothesis of a waterborne origin, as norovirus GI has been the most frequently genogroup implicated in water borne outbreaks (Maunula et al. 2005). This was the first study of a viral gastroenteritis outbreak among military in the Portuguese Army and from the lessons learned appropriate actions were taken. Since then, the distribution of potable water during military exercises has been reinforced and we had not detected another waterborne outbreak in a military exercise context. In addition, finding that different pathogenic agents and several transmission routes

may co-occur in a single outbreak request a detailed epidemiological investigation that characterizes the dynamics of the outbreak and the associated clinical symptoms that should be analyzed in context of the laboratory results to determine causality.

The second gastroenteritis outbreak investigated in this study occurred in April 2015 in a Portuguese Army base (Lopes-João et al. 2017). Stool analysis of seven cases were all positive for norovirus GI.9 presenting 100% nucleotide sequence homology, suggesting that this norovirus strain was the most probable cause of the outbreak. The investigation of the source of infection was complicated because affected soldiers ate in different base canteens, each with its own team of food handlers. Moreover, the usage of food samples on bacteriological analysis (traditionally the first pathogens to be searched) did not left any food or water available for virologic analysis that could have allowed a definite conclusion on the outbreak origin. Poor hygiene in manipulation of food was suspected in canteen A based on the presence of *Clostridium perfringens* in the meal and its absence in the raw materials used to prepare it. However, this outbreak could not be ascribed to poor hygiene in food handling, since each canteen had its own staff and the bacteriological analysis of the food served at canteen B indicated good microbiological quality. It would be tempting to attribute the norovirus GI.9 transmission to an infected food handler, but this is highly unlikely since different cooking teams prepared the food (one in each unit/canteen) and soldiers were not allowed to eat in canteens from other companies. Based on all the data, it was tempting to speculate that this GI.9 norovirus outbreak might have been waterborne and that the groundwater that was supplied to the canteens could be the source of the norovirus contamination. This hypothesis could not be confirmed, since the analyzed water was only sampled at the end of the outbreak, after the alert and adjustment of disinfection measures. This study showed the importance of continuous epidemiological surveillance and swift intervention in military settings to enable infection source identification. With the improvement of the base groundwater treatment no further cases were detected. Nevertheless, this study call attention for the need of considering the water as a norovirus source in outbreak investigations and illustrates the impact of norovirus GI.9 in the morbidity of military personnel.

The two above outbreaks were both caused by norovirus GI, namely GI.3 and GI.9 genotypes. These genotypes were previously found in drinking water or were related to waterborne outbreaks in non-military environments (Maunula et al. 2005; Nenonen et al. 2012; Lun et al. 2018). In fact, norovirus GI strains have been more often implicated in waterborne outbreaks than GII, possible due to a higher stability of GI strains in water than GII strains (Matthews et al. 2012). Conversely norovirus GII strains have been more associated with foodborne and person-to-person transmission (Lysén et al. 2009; Verhoef et al. 2010).

The third investigation was focused on four norovirus outbreaks that were reported to the surveillance system of the Portuguese Army between October 2015 and October 2017

(Lopes-João et al. 2018). Phylogenetic analysis showed that the noroviruses involved in these outbreaks belonged all to genogroup II, but to different genotypes, namely GII.17, GII.Pe-GII.4 Sydney 2012, GII. P2-GII.2 and GII.P16-GII.2, highlighting the genetic diversity of the circulating norovirus strains. These outbreaks appeared in geographically distant sites in the country and occurred in stationed military personnel, as well as, during military exercises, affecting almost 100 soldiers of which 30 had to receive hospital treatment due to the severity of symptoms. The considerable morbidity caused by these four norovirus outbreaks in the military personnel and its impact in the army effectiveness and force readiness during this two-years surveillance period give a broader insight of the impact of noroviruses in the Portuguese Army. Food handlers were the prime suspects of the origin of two of these outbreaks, reinforcing the importance of the early detection and report of gastrointestinal illness in these workers as an important outbreak prevention measure. In fact, a food handler showing gastroenteritis symptoms should be readily suspended of his duties in the food sector and remain away for at least a few days after the symptoms remission. Infected individuals shed the virus primarily in stool, but also in vomitus. Shedding of the virus may actually occur after symptom resolution, for a period of at least 2 to 3 weeks which complicates control recommendations (Atmar et al. 2008).

In the other two outbreaks a transmission through direct person-to-person contact or through contaminated fomites was suspected, as no epidemiological or laboratory data linked these outbreaks to food, water or even to a food handler. Outbreak investigations frequently implicate vomiting as a major transmission risk as half of all subjects with symptomatic infection experienced vomiting and the average subject shed is $1.7x10^8$ genomic equivalent copies (GEC)/ml in emesis (Kirby et al. 2016). Vomiting is also more likely to result in significant environmental contamination leading to transmission through fomites and airborne droplets (Kirby et al. 2016; Xiao et al. 2017). In fact, one of the gastroenteritis cases reported in these outbreaks was a soldier that got sick after cleaning the vomitus of another colleague. This illustrates the need of individual protection equipment cleaning facilities contaminated with stools or vomitus of an infected person. The swift and vigorous cleaning and disinfection procedures of the food sectors and sanitary facilities, after the detection of the outbreak, have played an important role in its rapid control (Hall et al. 2011). Curiously, these outbreaks lasted for a maximum of four days, which represents a short epidemiological curve when compared to reports from outbreaks in other military settings that extended for weeks (Mccarthy et al. 2000; Ahmed et al. 2012; Ho et al. 2015). Rapid implementation of preventive measures may have contributed to the short duration of the outbreaks.

The last investigation of these works reported a simultaneous gastroenteritis outbreak that was detected in three different bases of the Portuguese Army, in December 2017 (Lopes-João et al. 2019). The outbreak was caused by GII.P16-GII.4 Sydney norovirus, a recombinant

strain that was found in the stools of the sick soldiers from the three units. This outbreak revealed an interesting epidemiological profile since it affected three Army Units simultaneously where, all cases occurred in a 2-day period and with similar attack rates that were relatively low as compared to typical norovirus outbreaks. This profile suggested that norovirus was introduced at the same moment in the three units and had a common source of infection. Although it was not possible to identify the exact source of the outbreak the lettuce salad was suspected since it was the only item consumed raw and had been served in the three units two days before the outbreak onset. Moreover, the high numbers of enterobacteria detected in lettuce salad revealed that it was improperly disinfected. In fact, lettuce salad has been frequently implicated in the origin of norovirus outbreaks in military settings (Grotto et al. 2004; Wadl et al. 2010; Lopes-João et al. 2018) and has been shown to be an important source of transmission of noroviruses (Ethelberg et al. 2010; Gao et al. 2016).The lesson taken from this outbreak was that the centralization of food processing in main cuisines in the Army, although economically appealable, may present a challenge for the food safety and defense systems, with the potential exponentiation of case numbers in foodborne outbreaks and increased impact in operational effectiveness. This outbreak also showed the vulnerability of food supply chains to agents like noroviruses and reinforced the relevance of the correct disinfection procedures of raw vegetables. On the other hand, the rapid control of the outbreak was noteworthy. Immediate and strong control measures were implemented has soon has the outbreak was detected in both the food sector, medical centers and sanitary facilities and no secondary transmission was detected, which may partly explain the low attack rate of the outbreak.

Altogether, these four investigations allowed detailing the origin, the main transmission routes and to ascertaining the impact of norovirus gastroenteritis in the Portuguese Army. Although the real incidence of norovirus gastroenteritis in the Portuguese Army could not be precisely estimated it was uncovered that noroviruses were responsible for the majority of acute gastroenteritis outbreaks as well as for a considerable number of sporadic cases of gastrointestinal illness in 5-year period 2013-2017. This is in accordance with published data from different western countries Armies (Mccarthy et al. 2000; Delacour et al. 2010; Brooks et al. 2018).

It is interesting to note that different norovirus genotypes were detected in these outbreaks illustrating the high diversity of circulating strains. The genotype pattern seemed to follow the emergence of noroviruses strains and recombinant variants that contemporaneously circulated in Europe and worldwide (Cannon et al. 2017; Ruis et al. 2017; van Beek et al. 2018). In fact, norovirus GII.4 Sydney 2012 that was responsible for the second outbreak of February 2013 in Portuguese Army, also caused the gastroenteritis outbreak that occurred in the same month in a group of students in the North of Portugal (Mesquita et al. 2014). Similarly,

the genotype GII.17 detected in the outbreak of October 2015 was also the predominant one circulating in France in the winter of 2015/2016, (Bidalot et al. 2017) and GII.P16-GII.2 the recombinant variant, responsible for the outbreak of January 2017 was described first in France in November 2016 (Bidalot et al. 2017). It is tempting to speculate that a transboundary origin from France might have occurred since there is a strong traditional Christmas travelling of Portuguese emigrants in France back to Portugal to reunite with their families during the festivities. A considerable number of different genotypes of norovirus GII were successively identified during this five-year period corroborating the recombination capacity within this genogroup and highlighting that herd immunity at population level may have been a determinant of the molecular epidemiology of these norovirus outbreaks.

Although the origin of the eight norovirus outbreaks was difficult to confirm a foodborne or waterborne transmission was strongly suspected in five of them (62.5%). This is in line with a study that found that food- and waterborne transmission accounted for about 65% of the norovirus outbreaks globally (Matthews et al. 2012). Moreover, we found that norovirus was the most frequent etiologic agent responsible for foodborne illness in the Portuguese Army, being identified in 50% (5/ 10) of the foodborne or waterborne gastroenteritis outbreaks, and responsible for 41% (133/ 321) of the disease cases. Nevertheless, the number of cases may be underestimated as in some of these outbreaks secondary transmission was suspected and the exact number of affected personnel was not possible to ascertain. Noroviruses have been also ranked as the most frequent causal agents of foodborne disease in USA, Canada and Australia, accounting for 58%, 62,5% and 30% of foodborne illness cases, respectively (Hall et al. 2005; Scallan et al. 2011; Thomas et al. 2013). In the annual summaries of foodborne outbreaks in the USA, reported by CDC, norovirus was the most common cause of confirmed single-etiology outbreaks accounting for 35.4% of the outbreaks and 42% of the cases, in the time period of 2013 to 2017 (CDC 2019).

During the study period the Portuguese Authorities, namely *Instituto Nacional de Saúde Dr Ricado Jorge* and *Direção Geral de Alimentação e Veterinária* had reported to EFSA nine foodborne norovirus outbreaks totalizing 352 cases (appendix). Noroviruses accounted for 8% (9/106) of the foodborne outbreaks registered in Portugal and 13% (352/2665) of the cases of foodborne illness. In the same period, the Portuguese Army registered five outbreaks associated with food or water that caused 133 cases and were responsible for 50% of all foodborne outbreaks and 41% of foodborne illness. These populations are not comparable, nor in size or in age structure, but our results enable us to infer that the notification rate is much higher in the Army then in the civilian community. The confined environment that characterizes the Armed Forces Units likely underlies a higher incidence of norovirus gastroenteritis outbreaks as compared to the general community. The outbreaks in the military were not

included in the Portuguese data reported to the European authorities but the data concerning the Army outbreaks will be shared and reported to the Portuguese Authorities.

Overall, the present study contributed to introduction and implementation of preventive measures regarding food and water safety and allowed the reinforcement of control actions that minimized the length of gastroenteritis outbreaks of viral origin. This work also entailed setting-up GOSS in the Portuguese Army that revealed to be an important tool allowing the diagnostic of gastrointestinal disease in an outbreak context and to better ascertain the impact of foodborne illness in the Portuguese Army. The Portuguese Army GOSS has also provided valuable epidemiologic, microbiologic and clinical information that is used to improve prevention measures and likely prevent new outbreaks.

V. CONCLUSIONS

The results obtained in the present thesis lead to the following conclusions:

1. Norovirus was the most common etiologic agent of acute gastroenteritis outbreaks in the Portuguese Army, accounting for the majority of outbreaks and disease cases;

2. Norovirus was the most frequent cause of food- and waterborne illness in the Portuguese Army;

3. Norovirus was frequently diagnosed in otherwise non-specific of sporadic gastrointestinal disease in the Portuguese Army;

4. Norovirus outbreaks in the Portuguese Army were caused by both genogroup I, associated to waterborne and genogroup II associated with foodborne outbreaks and person-to-person transmission; Outbreaks caused by GII clearly outnumbered the outbreaks caused by GI;

5. Norovirus GII outbreaks were most frequently caused by genotypes GII.4 and GII.2;

6. Norovirus genotypes GI.9, GII.17 and GII.16-GII.2 in Portugal have been reported for the first time in this study, reflecting the great genetic diversity of noroviruses and their circulation in the community;

7. Norovirus outbreaks occurred either in military exercise context or in military bases and showed relevant impact on force readiness and operational effectiveness.

This work had consequences at different levels:

1. Has contributed to the set-up the “Gastroenteritis Outbreak Surveillance System” in the Portuguese Army that have revealed to be an important tool allowing the diagnostic of gastrointestinal disease in an outbreak context and to evaluate the impact of foodborne illness in the Portuguese Army;

2. Has led to the implementation of molecular techniques for the diagnosis of norovirus infection;

3. It contributed to change preventive measures in the food sectors of the Portuguese Army and allowed the reinforcement of control actions that minimized the spread of gastroenteritis outbreaks of viral origin.

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APPENDIX

Numbers of foodborne and waterborne outbreaks and number of cases of all origins and caused by norovirus registered in Portugal and in the Portuguese Army between 2013 and 2017.

Foodborne and waterborne outbreaks								
Year	Portugal General population				Portuguese Army			
	Total outbreaks		Norovirus outbreaks		Total outbreaks		Norovirus outbreaks	
	outbreaks	cases	outbreaks	cases	outbreaks	cases	outbreaks	cases
2013	19	388	2	96	1	20	1	19
2014	25	902	5	243	0	0	0	0
2015	20	423	0	0	2	71	1	46
2016	24	629	1	7	2	66	1	20
2017	18	323	1	6	5	166	2	48
Total	106	2665	9	352	10	323	5	133

The data from Portugal were obtained from EFSA in Biological hazards reports from 2013 to 2017 (EFSA 2019), https:www.efsa.europa.eu, accessed in 11 July 2019.